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INHIBITION OF PITUITARY ACTH RELEASE: AN EXTRA-ADRENAL ACTION OF EXOGENOUS ACTH[1]

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ABSTRACT

Pituitary ACTH concentration is approximately doubled after administration of ACTH for one week to adrenalectomized rats, in confirmation of previous studies in intact animals. Attempts to determine the mechanism responsible for this extra-adrenal effect of exogenous ACTH yielded the following results: Pre-treatment with ACTH inhibits the pituitary ACTH depletion observed in adrenalectomized rats after application of a scalding stress. Similarly, ACTH pre-treatment blocks the fall in adrenal ascorbic acid obtained in intact control rats after the stress of ether anesthesia. The data are compatible with the hypothesis that the increase in pituitary ACTH concentration produced by exogenous ACTH is due to inhibition of endogenous ACTH release by the pituitary gland. The possibility that stimulation of ACTH synthesis or pituitary adsorption of exogenous ACTH may also occur after administration of ACTH cannot be excluded.

PREVIOUS studies (1) demonstrated that daily administration of ACTH gel to intact rats for one week resulted in a rise of 95% in pituitary ACTH concentration, whereas the administration of cortisone under identical conditions produced a 60% fall. Since the only commonly accepted effect of ACTH is stimulation of the adrenal cortex, ACTH administration might reasonably be expected to cause pituitary ACTH de-

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pletion similar to that obtained with cortisone, rather than the increase in pituitary ACTH actually observed. The present study was undertaken to determine the mechanism of this unexpected action of injected ACTH.

METHODS

All experiments were performed in male rats of the Sherman strain, 90–140 gm., kept in a constant temperature room for at least one week prior to use. Diet consisted of Purina laboratory chow and water *ad libitum*. Control animals received physiological saline injected subcutaneously once daily. ACTH-treated animals received a long-acting ACTH preparation, either ACTH gel or Zinc ACTH, 4 or 6 U.S.P. units, injected subcutaneously once daily. Adrenalectomized rats were given their first injection immediately after operation and were maintained with supplementary 1% saline in the drinking water. Scalding served as a severe stress and consisted of immersion to the neck in water at 70° C. for 5 seconds under ether anesthesia. Ether anesthesia alone was used as a mild stress and was administered under the standardized conditions described by Wells *et al.* (2). Adrenal ascorbic acid levels were determined by the method of Mindlin and Butler (3).

The pituitary glands of the experimental animals were extracted for ACTH according to the method of Birmingham *et al.* (4). These extracts were subsequently assayed for ACTH either singly or in pools by incubation with rat adrenal slices *in vitro* by a modification of the technique of Saffran and Schally (5) previously described (1).[4] Individual pituitary extracts from ACTH gel-treated rats in the first experiment were assayed once each against randomly selected extracts from saline-treated rats. Pooled pituitary extracts in the second and third experiments were assayed 2 to 5 times each against their appropriate controls. Pituitary ACTH concentrations in the various experimental groups are expressed, therefore, as per cents of their respective control concentrations. The data were calculated in the form of concentration of ACTH per mg. anterior pituitary tissue; however, values for the total ACTH content of the individual pituitary glands or pools did not differ from those presented for concentration. Statistical calculations were performed according to the methods of Bliss (7). The values presented in the various tables are mean potency ratios with their approximate standard errors (Antilog $\overline{M} \pm 2.303\ s_{\overline{M}}$ Antilog $\overline{M}$). Statistical comparisons were based on the mean log potency ratios and their log standard errors ($\overline{M} \pm s_{\overline{M}}$). The number of replicate assays performed is noted in each table when indicated, as is the number of degrees of freedom (DF) for error available for statistical comparisons. The technique of bioassay of ACTH as employed permitted discrimination of differences in ACTH potencies as statistically significant when in excess of approximately 20%.

RESULTS

Experiment 1. The effect of ACTH pre-treatment on pituitary ACTH concentration in the absence of the adrenal glands was determined by

[4] Recently, Roberts (6) has questioned the specificity of the response of rat adrenal slices *in vitro* to ACTH. His conclusion of non-specificity was based on the observation that plasma obtained from hypophysectomized rats, as well as intact and adrenalectomized animals, stimulated corticosteroid release *in vitro*. Similar studies in this laboratory revealed that a small increment in corticosteroid release from adrenal slices occurred following the addition of plasma. However, this effect was constant and unrelated to the quantity of plasma added, i.e., a dose-response relationship could not be demonstrated. It should be noted that the amounts of plasma employed to produce such an increment represent quantities 2000–6000 fold greater by weight than the amounts of pituitary tissue actually used in the *in vitro* bioassay of ACTH.

administration of either saline (control) or 4 U.S.P. units of ACTH gel for 7 days to adrenalectomized rats. Both groups were sacrificed 24 hours after the last injection. A significant increase in pituitary ACTH concentration to 192% of the control level was observed in the rats given ACTH gel (Table 1). This rise is consistent with that previously obtained in intact rats (1).

TABLE 1. EFFECT OF EXOGENOUS ACTH ON PITUITARY ACTH CONCENTRATION IN ADRENALECTOMIZED RATS

Treatment	No. of rats	Pituitary ACTH as % control level*	P
Saline control	9	100	
ACTH gel	9	192 ± 40	$< .02$

* Antilog $\overline{M} \pm 2.303\ s_{\overline{m}}$ (Antilog $\overline{M}$).

Experiment 2. Changes in pituitary ACTH concentration following ACTH administration in adrenalectomized rats were studied in greater detail after the injection of 4 U.S.P. units of ACTH gel daily for 8 days. Groups of 4 animals each were sacrificed immediately after the last injection (control) and at 2, 4, 6 and 24 hours thereafter. No change in pituitary ACTH concentration was obtained at 2, 4 or 6 hours after the last injection of ACTH in comparison to the control level (Table 2). A suggestive, but not statistically significant, decrease in pituitary ACTH was observed at the 2-hour interval. On the other hand, a significant increment above the control level was found 24 hours after the last injection of ACTH; this increase is equally significant when compared to the potencies obtained at the 2, 4 or 6 hour intervals. However, the possibility of a gradual accretion in pituitary ACTH prior to the sixth hour cannot be excluded entirely owing to the relative imprecision of the assay technique.

Experiment 3. The effects of pre-treatment with ACTH on pituitary ACTH concentration after the application of the severe stress of scalding were studied in 7 groups of adrenalectomized rats. Groups 1–3 were given saline daily and served as controls, whereas Groups 4 and 6 were given 4 U.S.P. units of Zinc ACTH daily and Groups 5 and 7 were given 6 U.S.P. units of ACTH gel daily. Each group was randomly divided into

TABLE 2. CHANGES IN PITUITARY ACTH CONCENTRATION IN UNSTRESSED ADRENALECTOMIZED RATS INJECTED WITH ACTH

Time after last ACTH injection (hours)	No. of rats	Pituitary ACTH as % control level*	P	No. of assays	DF
0	4	100			
2	4	81 ± 15	n.s.	2	16
4	4	93 ± 18	n.s.	2	16
6	4	102 ± 12	n.s.	5	29
24	4	148 ± 20	$< .01$	3	16

* Antilog $\overline{M} \pm 2.303\ s_{\overline{m}}$ (Antilog $\overline{M}$).

unstressed and stressed subgroups after 8 injections had been administered. The scalding stress was applied to Groups 1–5 two hours after the last injection and both the unstressed and stressed subgroups were sacrificed simultaneously 4 hours after stress. The same procedure was applied to Groups 6 and 7 twenty-four hours after the last injection of ACTH.

Scalding produced consistent and significant depletions in pituitary ACTH concentration in the saline-treated controls (Groups 1–3) to between 67 and 72% of the respective unstressed levels (Table 3) with a mean of 70%. On the other hand, application of the identical stress to rats 2 hours after the last injection of Zinc ACTH (Group 4) resulted in a

TABLE 3. EFFECTS OF EXOGENOUS ACTH ON PITUITARY ACTH CONCENTRATION AFTER SCALDING STRESS IN ADRENALECTOMIZED RATS

Group	Treatment	No. of rats Stressed / Unstressed	Pituitary ACTH as % unstressed level*	P	No. of assays	DF
1	Saline	6/5	72 ± 10	< .05	3	36
2	Saline	6/5	72 ± 6	< .01	3	24
3	Saline	3/4	67 ± 10	< .01	4	20
	6 Hours After					
4	Zinc ACTH	6/5	122 ± 10	< .05	3	24
5	ACTH gel	3/4	88 ± 11	n.s.	4	31
	24 Hours After					
6	Zinc ACTH	3/3	80 ± 11	n.s.	3	24
7	ACTH gel	4/3	84 ± 20	n.s.	3	28

* Antilog $\overline{M} \pm 2.303\ s_{\overline{m}}$ (Antilog $\overline{M}$).

significant rise in pituitary ACTH concentration to 122% of the unstressed level—an effect which differs strikingly from the stress-induced pituitary ACTH depletion observed in saline-treated animals ($P < .01$). Rats pretreated with ACTH gel (Group 5) and scalded 2 hours after the last injection showed a pituitary ACTH concentration of 88% of the unstressed level, a difference which is not statistically significant. The possibility remains that a relatively small fall in pituitary ACTH may have occurred within the limits of discrimination of the assay technique. However, the post-stress potency is significantly higher than that obtained after stress in the saline-treated controls ($P < .05$). Groups 6 and 7, stressed 24 hours after the last injection of Zinc ACTH or ACTH gel respectively, also showed no significant difference between unstressed and stressed pituitary ACTH concentrations. However, the post-stress values do not differ significantly from those obtained in the saline-treated controls (Groups 1–3).

Experiment 4. The effects of ACTH pre-treatment on pituitary ACTH release after a mild stress was studied in intact rats after injection of either saline or 4 U.S.P. units of Zinc ACTH daily for 3 days. Twenty-four hours after the last injection, each group of animals was divided into unstressed and stressed subgroups and the latter subjected to ether anesthesia under

standardized conditions. One hour later all the animals were sacrificed. The stress of ether anesthesia produced a significant fall of 30% in adrenal ascorbic acid in intact control animals given saline (Table 4). On the other hand, no change in adrenal ascorbic acid was observed after stress in rats pre-treated with Zinc ACTH. To determine whether adrenal responsiveness to ACTH remained intact after pre-treatment with Zinc ACTH, hypophysectomized rats were similarly pre-treated with Zinc ACTH for 3 days starting 24 hours after operation. Twenty-four hours after the last injection, the left adrenal gland was removed under Nembutal anesthesia and 4 milliunits of aqueous ACTH injected intravenously. One hour later

TABLE 4. EFFECT OF EXOGENOUS ACTH ON ADRENAL ASCORBIC ACID AFTER ETHER STRESS IN INTACT RATS

Treatment	Subgroup	No. of rats	Adrenal ascorbic acid (mg./100 gm.)*	P
Saline	Control	10	441 ± 26	< .001
	Stressed	10	310 ± 17	
Zinc ACTH	Control	9	410 ± 22	n.s.
	Stressed	10	413 ± 18	
Hypophysectomy and Zinc ACTH	Control (Left Adrenal)	5	321 ± 14	< .001
	ACTH (i.v.) (Right Adrenal)	5	221 ± 7	

* Mean ± Standard Error of the Mean.

the right adrenal gland was removed. A 31% depletion of adrenal ascorbic acid resulted after ACTH injection, suggesting normal adrenal responsiveness.

DISCUSSION

The finding that ACTH administration produced a rise in pituitary ACTH concentration in adrenalectomized rats comparable to that observed previously in intact rats indicates that this effect of exogenous ACTH does not require mediation by the adrenal gland. A further increase in pituitary ACTH concentration was observed during the 24-hour period following the eighth daily injection of ACTH. The major portion of this rise occurred between the sixth and twenty-fourth hours with no apparent change in pituitary ACTH concentration prior to the sixth hour.

Three hypotheses may be invoked to explain these data. An increased blood level of ACTH may 1) result in non-specific ACTH adsorption by the pituitary gland; 2) stimulate ACTH synthesis; or 3) inhibit endogenous ACTH release with accumulation of normally synthesized hormone.

The last hypothesis was selected for further investigation. A scalding stress produced definite pituitary ACTH depletion in control animals.

Pre-treatment with ACTH modified this effect when stress was applied 2 hours after the last injection. An absolute rise rather than a fall in pituitary ACTH was observed after scalding in rats given Zinc ACTH. Pituitary ACTH concentration in rats pre-treated with ACTH gel after stress was higher than that observed in stressed control animals. Conclusions concerning these findings are subject to several considerations. Pituitary ACTH concentration at any moment is a measurement of a dynamic balance between the rate of ACTH synthesis and the rate of ACTH release; a change in concentration represents, therefore, a shift in this balance and not a direct measurement of either process alone. Thus, a fall (or negative balance) in pituitary ACTH concentration, such as that obtained after scalding in control animals, indicates that more ACTH was was released than synthesized from the time of stress to the time of sacrifice in response to the stressful stimulus. The degree of depletion measured is only a minimal estimate of the total quantity of ACTH actually released since newly synthesized ACTH may have been stored and/or released concurrently. On the other hand, pre-treatment with ACTH resulted in varying degrees of positive ACTH balance after scalding. Less ACTH was released than was synthesized and stored in stressed rats pre-treated with Zinc ACTH. The balance observed after stress in rats given ACTH gel was equivocal but was also more positive than that obtained in stressed control animals. Inhibition of pituitary ACTH release by exogenous ACTH with retention of newly synthesized hormone is suggested by these findings. Apparently, Zinc ACTH produced more complete inhibition of ACTH release than did ACTH gel. This observation perhaps may be explained by differences in the rates of absorption of the two preparations from sites of injection. Neither pituitary adsorption of exogenous ACTH nor accelerated synthesis of ACTH as a result of ACTH administration alone are applicable as explanations because 1) ACTH administration in the absence of stress did not increase pituitary ACTH concentration during the first 6 hours after the last injection—the period in which study of the effects of stress were completed; and 2) either ACTH adsorption or accelerated synthesis of ACTH, if present, should have affected the unstressed and stressed subgroups equally and are excluded, therefore, by the experimental design itself. An alternative explanation of the data would require the new hypothesis that ACTH pre-treatment resulted in acceleration of synthesis of ACTH (or increased pituitary adsorption of exogenous ACTH) only in the presence of stress but had no such effect in the absence of stress. Of course, this hypothesis does not explain the rise in pituitary ACTH obtained after ACTH pre-treatment in unstressed animals (Expts. 1 and 2). Significant inhibition of ACTH release as a result of pre-treatment with either preparation of ACTH was not demonstrated when the severe stress of scalding was applied 24 hours after the last injection.

A specific test of the inhibitory effect of pre-treatment with ACTH on pituitary ACTH release 24 hours after the last injection was performed with the milder stress of ether anesthesia. Since measurements of pituitary ACTH concentration before and after stress permit only indirect evaluation of the amount of ACTH released, alterations in adrenal ascorbic acid were employed as a more direct index of endogenous ACTH release. The data indicate that inhibition of pituitary ACTH release was present 24 hours after the last injection of Zinc ACTH and are in agreement with the previous findings. Since administration of various adrenal steroids may also result in inhibition of ACTH release (8), the effects of exogenous ACTH obtained in intact rats might conceivably be explained by the action of steroids secreted as a result of ACTH stimulation. However, the dose of corticosterone, the major corticoid hormone of rat adrenal glands, required to block ACTH release after stress apparently exceeds that amount maximally produced by the rat (9).

The administration of a long-acting ACTH preparation results in a significant rise in pituitary ACTH concentration in the adrenalectomized as well as the intact rat. The data presented support the hypothesis that this increase in ACTH concentration is due to inhibition of endogenous ACTH release from the pituitary gland. The findings neither support nor specifically refute the possibility that adsorption of exogenous ACTH or stimulation of ACTH synthesis may also occur. No conclusion may be made concerning the locus of this extra-adrenal action of ACTH, such as the pituitary gland itself or higher centers in the hypothalamus. Furthermore, since relatively large doses of ACTH were employed, these findings may well represent a pharmacologic rather than a physiologic effect of ACTH.

ADDENDUM

Since this paper was submitted for publication, Hodges and Vernikos (*Nature* 182: 725, 1958) have reported that less ACTH was discharged in response to ether stress as blood ACTH concentration increased following adrenalectomy in rats. These findings represent additional evidence in support of the hypothesis that pituitary ACTH release is regulated by the circulating level of ACTH.

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STUDIES ON THE CONTROL OF CORTICOTROPHIN RELEASE

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ABSTRACT

Different substances known to occur in hypothalamic nuclei and neurones were studied as ACTH releasers (adrenal ascorbic acid depletion method) in normal rats, in hypophysectomized rats, in hypophysectomized rats bearing a functional pituitary graft in the anterior chamber of the eye, and in rats in which the ACTH discharge following non-specific stimuli had been blocked by administration of 9-alpha-fluoro-hydrocortisone.

Adrenaline, noradrenaline, acetylcholine, histamine, serotonin, Pitressin, Pitocin, lysine-vasopressin and synthetic oxytocin were found highly effective in normal animals; Euler's substance P did not produce adrenal ascorbic acid depletion in normal rats.

Acetylcholine, histamine, serotonin, Pitocin, synthetic oxytocin and adrenaline were ineffective when tested in the hypophysectomized-grafted animals; only Pitressin gave a significant fall in adrenal ascorbic acid concentration when directly applied to the grafted pituitary gland.

In the steroid-blocked rats Pitressin, Pitocin and lysine-vasopressin were active as ACTH releasers when given through intravenous injections; the intracarotid injection of small doses of Pitressin was also active in the blocked rats; the other stressors were inhibited after steroid administration.

These results are discussed in connection with the hypothesis that posterior pituitary principles could play a role in the physiological regulation of ACTH output.

THE view that hypophyseal portal vessels regulate anterior pituitary function by transmitting a hypothalamic humoral substance, has received support from the results of several anatomical and physiological studies. In confirmation of earlier observations on amphibians and rats (1, 2) Worthington (3) has recently shown that blood in the portal vessels of the mouse flows from the capillaries in the median eminence to the sinusoids of the pars distalis; moreover Landsmeer (4), Jewell (5) and Daniel *et al.* (6) have recently demonstrated in dogs that the blood reaching the neurohypophysis via the posterior lobe artery, may readily flow into the anterior lobe through an additional system of anastomoses.

Several suggestions have been put forward as to the nature of the postulated hypothalamic neuro-humor: adrenergic substances (7), histamine

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(8), serotonin (9), substance P (10), lipoid or lipoprotein present in hypothalamic extracts (11), etc.

In recent years attention was drawn to a possible relationship between the antidiuretic hormone (ADH) of the posterior pituitary system and ACTH secretion. This hypothesis is supported by the following evidence: a) extracts containing the antidiuretic activity of the posterior lobe are effective in inducing ACTH release in normal animals (12, 13, 14, 15), in hypophysectomized animals bearing a functional pituitary graft in the anterior chamber of the eye (16), and in rats with hypothalamic lesions which block the response to non-specific stimuli (17); b) a defect in ACTH release is correlated with diabetes insipidus in rats with hypothalamic lesions (17); c) the neurosecretory material stored in the neurohypophysis and containing ADH may be depleted by noxious stimuli which also induce ACTH release (18, 19); d) ADH and ACTH are discharged simultaneously after the exposure to stressful stimuli (20) and after the administration of several drugs (21, 22).

It seemed of interest to re-investigate *in vivo* the effect of different substances, known to occur in hypothalamic nuclei and neurones (10, 23, 24, 25), on ACTH release, using normal rats, hypophysectomized rats, hypophysectomized rats bearing a functional pituitary graft in the anterior chamber of the eye, and rats in which the ACTH discharge following non-specific stimuli had been blocked by previous administration of adrenocortical steroids.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, weighing 150–200 gm., were used throughout these experiments. All animals were kept at a constant room temperature of 26–28° C.; they were given a 5% glucose solution to drink and maintained on a stock diet. The ACTH-releasing effect of adrenaline-HCl, noradrenaline-HCl, acetylcholine-HCl, histamine acid phosphate, serotonin creatine-sulphate, substance P,[2] commercial, purified and synthetic antidiuretic and oxytocic hormones (Pitressin, Pitocin, Syntocinon and lysine-vasopressin[3]) was studied in normal, hypophysectomized, hypophysectomized-grafted and corticoid-blocked rats. The ACTH-releasing effect of the different drugs was evaluated by means of adrenal ascorbic acid depletion (26).

Normal rats received the different drugs intraperitoneally, the control group being injected with NaCl 0.9% solution. The hypophysectomized rats were prepared by the parapharyngeal approach and used in the ascorbic acid depletion test 24 hours later. The hypophysectomized-grafted rats were prepared according to a procedure previously described (16) and used 1 month after grafting. The study of the modifications induced by pituitary transplantation on endocrine glands weight and morphology forms the subject of separate communications (16, 27); it is worth anticipating that the adrenal weight

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[2] Two samples of substance P were obtained through the courtesy of Professor Gaddum (horse gut, 13.5 U./mg.) and of Professor Von Euler (cow gut, 100 U./mg.).

[3] Pitressin, Lot #31846 and Pitocin, Lot #209050 were kindly supplied by Dr. D. A. McGinty of Parke, Davis and Co.; Syntocinon was kindly supplied by Dr. A. Cerletti of Sandoz Ltd., Basle; lysine-vasopressin was obtained through the courtesy of Dr. G. A. Overbeek of N. V. Organon, Oss.

of the grafted animals was found lower than in intact controls, although much higher than in complete hypophysectomized animals. In the grafted rats the different drugs, dissolved in 0.1 cc. of saline, were directly injected into the anterior chamber of the graft-containing eye.

The hydrocortisone inhibited rat has been recently proposed for the study of hypophysiotropic substances, because of its failure to react with ascorbic acid depletion to unilateral adrenalectomy (28). Casentini *et al.* (29) reported that several other natural or synthetic steroid which exhibit adrenocortical activity block ACTH discharge after ether anesthesia and unilateral adrenalectomy. 9-alpha-fluoro-hydrocortisone proved to be 10 times as potent as hydrocortisone; this steroid was chosen for the experiments of the present paper. The steroid was administered intraperitoneally as a suspension in 0.3 cc. of an aqueous system of 0.9% NaCl; 0.4% Tween 80; 0.5% carboxymethylcellulose; 0.9% benzyl alcohol; the steroid inhibited rats were used in the ascorbic acid depletion test 4–6 hours after the injection of the blocking agent. In these rats, as well as in the hypophysectomized ones, the different drugs were usually given intravenously; in some experiments the blocked-rats received intracarotid injections. In hypophysectomized, in hypophysectomized-grafted and in steroid-blocked rats, right adrenalectomy was performed under ether anesthesia, to obtain a control level of ascorbic acid; the test-drug was administered immediately thereafter.

All animals were killed by decapitation 1.5 hours after treatment and the left adrenal was immediately removed. Adrenal glands were rapidly dissected free of fat and weighed on a torsion balance; their content of ascorbic acid was then estimated by the method of Roe *et al.* (30).

At the time of the autopsy the sella turcica of the hypophysectomized and hypophysectomized-grafted rats was examined with a magnifying glass for hypophyseal fragments, completeness of hypophysectomy being later confirmed by microscopic examination of serial sections through the sellar region.

The significance of the differences between the means obtained was calculated by Student's "t" test.

RESULTS

Normal rats

Confirming earlier observations the intraperitoneal injection of adrenaline (31), noradrenaline (32), acetylcholine (22), histamine (32), serotonin (9), Pitressin (13), Pitocin (13) was followed by a highly significant fall in adrenal ascorbic acid concentration ($P < 0.001$) in normal animals (Table 1); lysine-vasopressin and synthetic oxytocin also showed marked ACTH releasing activity in normal rats (Table 1). Two different samples of substance P were completely ineffective in the ascorbic acid depletion test (Table 1); this last result differs from Swingle *et al.* (33).

Hypophysectomized rats

Pitressin, Pitocin and lysine-vasopressin were tested in hypophysectomized rats in order to exclude the possibility of contamination with ACTH.

Table 2 shows that Pitressin, Pitocin and lysine-vasopressin, at the dose administered, do not induce significant ascorbic acid depletion in hypophysectomized rats.

TABLE 1. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN NORMAL RATS

Treatment	No. of rats	Adrenal ascorbic acid concentration (mg./100 gm. adrenal) Mean ±S.E.*	Ascorbic acid depletion from controls (mg./ 100 gm. adrenal)	P (Fisher's table)
NaCl 0.9% i.p.†	26	411 ± 8		
Adrenaline 0.05 mg.i.p.	6	304 ±46	−107	<0.001
Noradrenaline 0.05 mg.i.p.	11	307 ±14	−104	<0.001
Acetylcholine 0.20 mg.i.p.	10	331 ±13	− 80	<0.001
Histamine 0.20 mg.i.p.	18	345 ± 7	− 66	<0.001
Serotonin 0.05 mg.i.p.	6	299 ±42	−112	<0.001
Substance P (horse) 10 U.i.p.	6	427 ±43	+ 16	
Substance P (cow) 10 U.i.p.	6	436 ±28	+ 25	
Pitressin 0.3 U.i.p.	12	302 ±14	−109	<0.001
Pitocin 0.3 U.i.p.	23	358 ±12	− 53	<0.001
Lysine-Vasopressin 0.3 U.i.p.	18	311 ± 8	−100	<0.001
Synthetic oxytocin 0.3 U.i.p.	23	358 ±16	− 53	<0.005

* S.E. = Standard Error of the mean.
† i.p. = intraperitoneally.

TABLE 2. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY PITRESSIN, PITOCIN AND LYSINE-VASOPRESSIN IN HYPOPHYSECTOMIZED RATS

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ±S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9% i.v.†		+56 ±12	
Pitressin 0.3 U. i.v.	6	+16 ±36	
Pitocin 0.3 U. i.v.	6	−35 ±17	>0.20
Lysine-vasopressin 0.3 U. i.v.	5	+11 ±12	

* S.E. = Standard Error of the mean.
† i.v. = intravenously.

Hypophysectomized-grafted rats

After severance of the pituitary stalk and transplantation of the pituitary to a distant site in the body, the rat no longer responds with adrenal ascorbic acid depletion to the stress of ether anesthesia and unilateral adrenalectomy (Table 3); in the grafted rats acetylcholine, histamine, serotonin, Pitocin and synthetic oxytocin were completely ineffective as ACTH-releasers, when injected into the anterior chamber of the graft-containing eye.

Adrenaline gave a positive response in one rat, a result which cannot be considered as significant.

Among the drugs tested only Pitressin gave a significant fall ($P < 0.005$) in adrenal ascorbic acid concentration, when directly applied to the grafted pituitary gland.

Steroid blocked rats

A. *Intravenous injections.* The sensitivity of the steroid inhibited rat to stressful stimuli and to ACTH administration has not been fully evaluated so far. To make certain that the site of the blocking action of the

TABLE 3. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN HYPOPHYSECTOMIZED-GRAFTED RATS: LOCAL ADMINISTRATION TO THE GRAFT-CONTAINING EYE

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ± S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9%	6	+59 ± 12	
Adrenaline 0.005 mg.	4	−27 ± 29	N.S.
Acetylcholine 0.03 mg.	10	+ 6 ± 11	
Histamine 0.05 mg.	5	+34 ± 15	
Serotonin 0.005 mg.	5	+37 ± 15	
Pitressin 0.01 U.	6	−56 ± 11	<0.005
Pitocin 0.01 U.	6	−15 ± 13	N.S.
Synthetic oxytocin 0.01 U.	6	− 1 ± 20	

* S.E. = Standard Error of the mean.

steroid was not at the adrenal level, the adrenal ascorbic acid response of 9-alpha-fluoro-hydrocortisone blocked rats to different intravenous dosages of ACTH was examined.

Figure 1 shows that in blocked rats a linear log-dose relationship can be obtained with doses of ACTH ranging from 1 mU. to 16 mU. per rat; the ACTH-sensitivity of the steroid-blocked rats appears to be similar to that of normal animals but less than that of hypophysectomized animals (34).

When testing the different drugs in the 9-alpha-fluoro-hydrocortisone inhibited rats (Table 4) it became apparent that many stressors were completely ineffective as ACTH releasing agents. Only Pitressin, Pitocin and lysine-vasopressin could overcome the blockade, but only in the case of Pitressin and lysine-vasopressin the degree of significance was similar to that obtained in the normal animals.

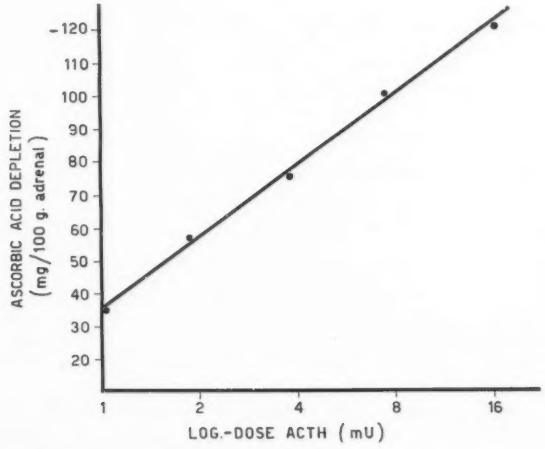

FIG 1. Adrenal ascorbic acid depletion induced by intravenous injections of ACTH in 9-alpha-fluorohydrocortisone blocked rats.

TABLE 4. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN RATS BLOCKED BY 9-ALPHA-FLUOROHYDROCORTISONE (0.9 MG./100 G.B.W.)

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ± S.E.*	P (Fisher's table)
Unilateral adrenalectomy	6	+29 ± 16	
Unilateral adrenalectomy plus			
NaCl 0.9% i.v.†	6	+18 ± 7	
Adrenaline 0.05 mg.i.v.	6	− 4 ± 17	
Noradrenaline 0.05 mg.i.v.	6	+31 ± 14	
Acetylcholine 0.20 mg.i.v.	12	−36 ± 22	>0.1
Histamine 0.20 mg.i.v.	6	−25 ± 15	>0.1
Serotonin 0.05 mg.i.v.	9	−27 ± 19	>0.1
Substance P (horse) 10 U.i.v.	6	+28 ± 15	
Substance P (cow) 10 U.i.v.	16	+19 ± 23	
Pitressin 0.3 U.i.v.	19	−70 ± 12	<0.001
Pitocin 0.3 U.i.v.	10	−41 ± 17	<0.05
Lysine-vasopressin 0.3 U.i.v.	18	−78 ± 11	<0.001
Synthetic oxytocin 0.3 U.i.v.	7	−27 ± 29	>0.30

* S.E. = Standard Error of the mean.
† i.v. = intravenously.

B. *Intracarotid injections.* An objection against ADH as the possible physiological ACTH-stimulating substance was raised by the fact that rather high dosages of the hormone are usually required to elicit significant adrenal ascorbic acid depletions.

It seemed of interest to study whether the injection of Pitressin into the carotid blood, bringing the stimulating substance directly to the adenohypophysis, could allow a reduction of the amounts to be injected.

Table 5 shows that intracarotid injections of small doses of Pitressin are still active in the blocked rats; neither Pitocin nor synthetic oxytocin were active when given in small amounts by intracarotid injections in the blocked animals.

Figure 2 shows that Pitressin gives a linear log-dose relationship in doses ranging from 6 mU. (0.1 μg.) to 24 mU. (0.4 μg.) when given through intracarotid injections in 9-alpha-fluoro-hydrocortisone inhibited rats.

TABLE 5. ADRENAL ASCORBIC ACID DEPLETION PRODUCED IN 9-ALPHA-FLUORO-HYDROCORTISONE BLOCKED RATS BY INTRACAROTID INJECTIONS OF PITRESSIN, PITOCIN AND SYNTHETIC OXYTOCIN

Treatment	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal) Mean ± S.E.*	P (Fisher's table)
Unilateral adrenalectomy plus			
NaCl 0.9% i.c.†	10	− 3 ± 19	
Pitressin 0.012 U.i.c.	9	−87 ± 30	<0.025
Pitocin 0.012 U.i.c.	4	+16 ± 26	
Synthetic oxytocin 0.012 U.i.c.	5	−15 ± 38	>0.20

* S.E. = Standard Error of the mean.
† i.c. = intracarotid injection.

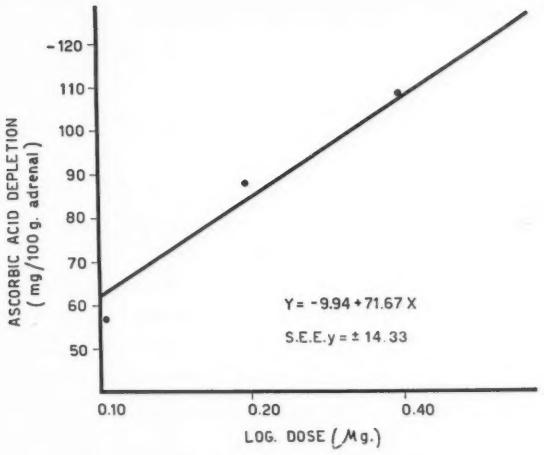

FIG. 2. ACTH releasing activity of intracarotid injections of Pitressin in 9-alpha-fluorohydrocortisone blocked rats. Pitressin = 60 U./mg.

DISCUSSION

Cheng *et al.* (35), McDermott *et al.* (36), Fortier (37) had shown in rats that adrenaline and histamine may induce ACTH release by the transplanted pituitary; similarly Hume (38) and Fortier *et al.* (39) observed in stalk-sectioned dogs and rabbits normal eosinopenic responses to adrenaline. In contradiction with these results our own observations seem to indicate that adrenaline and histamine (as well as acetylcholine and serotonin) induce ACTH release not through a direct action on hypophyseal glandular cells but probably through a nervous mechanism. This hypothesis is supported by many recent observations: Hume (40), Porter (41) and McCann (42) reported that hypothalamic lesions prevent the eosinopenia and the adrenal ascorbic acid depletion induced by adrenaline, histamine and Pitocin; Olling *et al.* (43), Wells *et al.* (44), Sevy *et al.* (45) and Van Peenen *et al.* (46) observed that certain nervous depressants (morphine, reserpine, chlorpromazine, etc.) inhibit the hypersecretion of ACTH in response to histamine, adrenaline and noradrenaline; Porter (41) and Nakayama (47) found electrical activation of hypothalamic nuclei after giving adrenaline and histamine; Schally *et al.* (48) and Gullemin *et al.* (49) found histamine, noradrenaline and synthetic oxytocin inactive in stimulating ACTH release from pituitaries incubated *in vitro*. An hypothalamic site of action for adrenaline and acetylcholine is also suggested by the studies on the mechanism of the antidiuretic action of these drugs (21, 22).

The ability of Pitressin to discharge ACTH when applied to the graft-containing eye is in agreement with an earlier observation (16) and shows that Pitressin directly acts on hypophyseal glandular cells; a similar con-

clusion was reached by McCann (42) working in hypothalamic-lesioned animals, and by Sevy *et al.* (45) working in chlorpromazine-inhibited rats. Sayers' observation (50) that sections through the brain stem at the upper level of the pons abolish the pituitary response to Pitressin is contradicted by the ACTH releasing activity shown by Pitressin when directly injected into the grafted pituitary.

The ability of steroids with adrenocortical activity to suppress the release of ACTH after unilateral adrenalectomy is in agreement with the results and conclusions of various workers (see 29 for references). Some recent results seem to suggest that adrenocortical steroids block ACTH secretion by acting upon nervous structures: in agreement with this hypothesis Courrier *et al.* (51) reported that labeled cortisone is concentrated *in vivo* by hypothalamic and posterior pituitary tissues; on the other hand McCann (42) Fulford *et al.* (52) and Ganong *et al.* (53) reported that the adrenal ascorbic acid response to unilateral adrenalectomy as well as the compensatory adrenal hypertrophy which normally follows unilateral adrenalectomy are suppressed by hypothalamic or stalk lesions. In contradiction to these results Fortier *et al.* (39) recently reported that stalk section in rabbits exerts little effect on the adrenal ascorbic acid depletion following unilateral adrenalectomy.

The experiments presented here provide two pieces of evidence of the fact that adrenal steroids normally act at some site proximal to the pituitary: 1) in the hypophysectomized grafted animals unilateral adrenalectomy was not followed by adrenal ascorbic acid fall in the remaining adrenal; 2) in agreement with previous observations (42, 54, 55, 56) Pitressin (the only agent which exhibited a direct stimulating activity on hypophyseal glandular cells when tested in the grafted animals) was found able to overcome the steroid blockade.

The results obtained injecting Pitressin into the carotid artery of corticoid-blocked rats seem to indicate that the pituitary is very sensitive to this stimulus; they also rule out the possibility of a direct action of Pitressin at the level of the adrenal cortex (57).

The ACTH-sensitivity of the 9-alpha-fluorohydrocortisone treated normal rats was found similar to that of untreated normal controls; this result, as well as Hodges' observations (58) in the DCA-treated normal rats, seems to rule out the possibility of a block of ACTH activity at the adrenal level; two recent reports (34, 59) deal with the reduced ACTH sensitivity of hypophysectomized corticoid-treated animals.

A few results had already shown that the corticoid-blocked rats no longer respond to histamine (54, 55, 60) and adrenaline (56, 61, 62). The results here presented extend these investigations and show that in the 9-alpha-fluoro-hydrocortisone inhibited rats the ACTH releasing activity of noradrenaline, acetylcholine and serotonin is also blocked; this last result was recently confirmed (56).

The 9-alpha-fluoro-hydrocortisone blocked rats, because of their specific response to posterior pituitary preparations (Pitressin, lysine-vasopressin and Pitocin), proved to be very useful for testing the ACTH releasing activity of hypophysiotrophic hypothalamic substances; the steroid blocked rats could also be employed in substitution of hypophysectomized animals in the biological assay of ACTH.

Our experiments though suggesting that a neurohumoral substance which stimulates the release of ACTH by acting directly at the hypophysial level is present in posthypophysial extracts do not identify it specifically; in particular they do not exclude the possibility that the effect observed after the administration of Pitressin and purified lysine-vasopressin could be due not to ADH itself but to a contaminant with specific ACTH releasing properties (CRF) as suggested by Saffran's (63) and Guillemin's *in vitro* experiments (49);[4] it should however be pointed out that it has recently been demonstrated that synthetic lysine-vasopressin also has an ACTH stimulating activity both *in vitro* and *in vivo* (65, 66, 67).

Evidence is now accumulating showing that posterior pituitary principles may play a role in the release of other pituitary trophic hormones, such as TSH (68, 69), STH (70), gonadotrophic hormones (71) and prolactin (72).

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THE ADRENOTROPIC ACTION OF HUMAN PREGNANCY PLASMA[1]

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ABSTRACT

Jailer and coworkers have reported that the plasma of pregnant women contained an adrenotropin which was distinct from ACTH; they showed that treatment of freshly hypophysectomized rats with pregnancy plasma partially prevented adrenal involution. We have found evidence suggesting that the adrenotropic action of pregnancy plasma is attributable to its content of chorionic gonadotropin. The gonadotropin appears not to act directly on the rat adrenal; instead it stimulates the interstitial cells of the testis, and their steroid secretory product in turn acts on the adrenal to produce a partial maintenance of adrenal weight.

JAILER and coworkers (1) have reported that plasma from pregnant women and from patients with Cushing's disease associated with adrenal hyperplasia contained an adrenotropic factor which was distinct from ACTH. This material partially prevented adrenal weight loss in hypophysectomized rats, but did not cause ascorbic acid depletion. We wondered whether the adrenotropic action of pregnancy plasma might be related to its content of chorionic gonadotropin (CG) and have made studies with this in mind. Our findings lead us to believe that CG in pregnancy plasma is responsible for the partial weight maintenance of the adrenal glands in hypophysectomized rats. However, CG does not act directly on the adrenal gland. It stimulates the interstitial cells of the testis in the hypophysectomized rats and the steroid secretory product of these cells then acts on the adrenals to produce a partial weight maintenance.

METHODS

Adrenotropic action was tested in individually caged male Sprague-Dawley rats hypophysectomized on the 40th day of life. Animals were obtained from the Hormone Bioassay Laboratories, Chicago, except where noted. Hypophysectomized female rats, intact male rats, and hypophysectomized, gonadectomized male rats were used as indicated in certain experiments. Injections of test material were begun on the 41st day and given subcutaneously in a volume of 0.3 cc. three times daily for 8 days (once daily for testosterone propionate in sesame oil). The rats were then killed by ether anesthesia and the two adrenals dissected and weighed together. In some cases testicular and adrenal tissues were fixed in 10% formalin for histological section. There were 6–10 indi-

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vidually caged animals in each treatment group. In these and in the other animal experiments mentioned subsequently, animals were assigned to treatment groups by random numbers, and the data were collected without knowledge of the treatment involved.

Chorionic gonadotropin was assayed by the rat ovarian hyperemia method as described by Albert and Berkson (2). One cc. of test material was injected subcutaneously into 30 to 50 gm. intact female rats; plasma samples were diluted at least tenfold with saline because of the inhibiting effect of higher concentrations of plasma in this test. Four hours after injection the animals were killed by ether anesthesia and the ovaries inspected. A dose response curve to standard chorionic gonadotropin in normal saline was plotted on semi-log paper. Our dose response curve was not identical with that obtained by Albert and Berkson, but the response was linear over the range of 16 to 85% positive responses (2–8 I.U. CG). The linear portion of the curve was plotted using data from 110 animals in 10 experiments. Unknown materials were diluted so that the response to one or more dilutions fell within the linear range. A minimum of 5 animals were tested at each dosage for both unknown and standard materials.

ACTH activity was measured by adrenal ascorbic acid depletion in hypophysectomized rats as described by Sayers and coworkers (3), using an Armour preparation with potency stated in international units as a standard. Five animals per group were used, each animal serving as its own control.

Blood from pregnant women was collected in heparin usually within 15 minutes of delivery, iced immediately, and centrifuged in a refrigerated centrifuge. The supernatant plasma was removed and frozen. Pools of plasma were prepared by combining individual specimens.

Chorionic gonadotropin, (Ayerst) was supplied as a dry powder prepared from human pregnancy urine. The stated potency was 1225 I.U./mg. We have used this material as our standard.

RESULTS

On the basis of the ovarian hyperemia assay, 4 maternal plasma pools contained from 22 to 58 I.U./cc. of chorionic gonadotropin and all partially prevented adrenal weight loss in hypophysectomized rats (Table 1A). When maternal plasma pools were diluted, the minimum effective dose preventing complete adrenal weight loss contained between 0.5 and 2.7 I.U./cc. CG. Two samples of pooled human plasma obtained from the blook bank contained no measurable CG activity (sensitivity limit 2 I.U./cc). and had no effect on the rat adrenal weights. However, the addition of chorionic gonadotropin to bank plasma in amounts between 11 and 165 I.U./cc. produced the same effect on the adrenals as did the undiluted maternal plasma pools. Smaller amounts of CG were then added to the bank plasma to determine the minimum effective dose, which was found to contain between 0.5 and 1 I.U./cc. CG. As may be seen from Table 1A, similar amounts of CG occurring naturally in maternal plasma or added to bank plasma produced similar effects on adrenal weight.

For purposes of comparison, gonadotropic substances from other sources were tested both for CG activity and prevention of adrenal weight loss. Sheep pituitary luteinizing hormone (LH) (Armour) containing 1 mg. Armour standard per cc. was found to be equivalent to 8 I.U./cc. CG in the ovarian hyperemia assay. This material was effective in preventing adrenal

TABLE 1

Material	CG= I.U./cc.	Adrenal weight (mg)	
		Mean	Std. error of mean
A. *The relationship of adrenal weights in hypophysectomized rats to the dosage of chorionic gonadotropin (CG) or its equivalent as measured in the rat ovarian hyperemia assay*			
Controls			
Saline	0	15.1	0.6
Non-injected	0	14.8	1.3
Maternal plasma pool			
1	58	21.8	0.7
2	46	20.7	1.2
3	25	21.3	0.5
4	22	21.0[a]	1.0
1 (1:10 dilution)	5.8	17.3	0.7
2 (1:10 dilution)	4.6	16.1	0.5
5 (1:10 dilution)	2.7	18.9	0.6
5 (1:50 dilution)	0.5	16.3	0.4
Bank plasma pool			
1	0*	14.9[b]	0.6
2	0*	15.9[c]	1.0
Chorionic gonadotropin added to bank plasma	165	20.0	0.6
	17	20.6	0.5
	11	22.0	0.8
	11	20.8	0.7
	3.3	19.1[d]	0.6
	1	19.2	0.6
	0.5	16.0	1.0
	0.05	14.2	0.6
Luteinizing hormone	8	20.8	1.0
Equine gonadotropin	7	19.7	1.0
B. *The relationship of adrenal weights in hypophysectomized rats to the dosage of ACTH*			
	ACTH I.U./cc.		
ACTH	0.28	21.8	0.6
	0.083	19.4[e]	0.5
	0.028	17.0	0.6
Bank plasma Pool 2	0	15.9[f]	1.0

[a–b,c–d,e–f] Test for significant difference by analysis of variance: $P < 0.01$ in each of these pairs.

* Sensitivity limit: 2 I.U./cc.

weight loss to the same extent as a corresponding dose of CG. Similarly, equine gonadotropin (Ayerst) in concentration of 150 I.U./cc. was equivalent to 7 I.U./cc. CG in the ovarian hyperemia assay and was also effective in preventing adrenal weight loss.

ACTH produced no ovarian hyperemia in the assay for CG, but, as expected, it was effective in preventing adrenal weight loss in hypophysectomized rats (Table 1B).

Histological sections of adrenal from hypophysectomized rats showed that maternal plasma (Fig. 3), CG (Fig. 5), LH, and ACTH (Fig. 7) pro-

duced a wider cortex than that seen in untreated animals (Fig. 1). The results of these various treatments were indistinguishable in hematoxylin and eosin stained sections. In Sudan stained sections, however, only ACTH treatment (Fig. 13) was associated with a fairly uniform distribution of fat-staining material, and disappearance of the sudanophobic zone (cf. Fig. 10, 11, 12 and 14). Histological sections from the testes likewise revealed a difference between the effect of ACTH (Fig. 8) and that of the other agents. Maternal plasma (Fig. 4), CG (Fig. 6) and LH maintained the interstitial cells of the testes, whereas ACTH did not.

Injections of bank plasma in control animals resulted in a thinning of the adrenal cortex with atrophy and hypoplasia most pronounced in its central portion. A sudan-free zone in the fascicular zone was present; the interstitial cells of the testes were not maintained.

Neither maternal plasma nor CG contained ACTH activity as measured by adrenal ascorbic acid depletion (Table 2). Equine gonadotropin and sheep pituitary LH produced slight depletions in adrenal ascorbic acid; the effect of each was less than that produced by 0.0011 I.U. ACTH.

Neither CG nor maternal plasma prevented adrenal involution in either hypophysectomized *female* rats or in hypophysectomized *and gonadectomized* male rats (Table 3A and B). However, partial adrenal weight maintenance could be effected in hypophysectomized gonadectomized male rats by the sole administration of testosterone propionate (Table 3C). These rats were of the same strain but were obtained from a different source. The degree of weight maintenance and the histological effects on the adrenal were identical with those previously obtained with CG and maternal plasma in hypophysectomized male rats with intact testes.

CG did not affect adrenal weights in intact male rats (Table 3D).

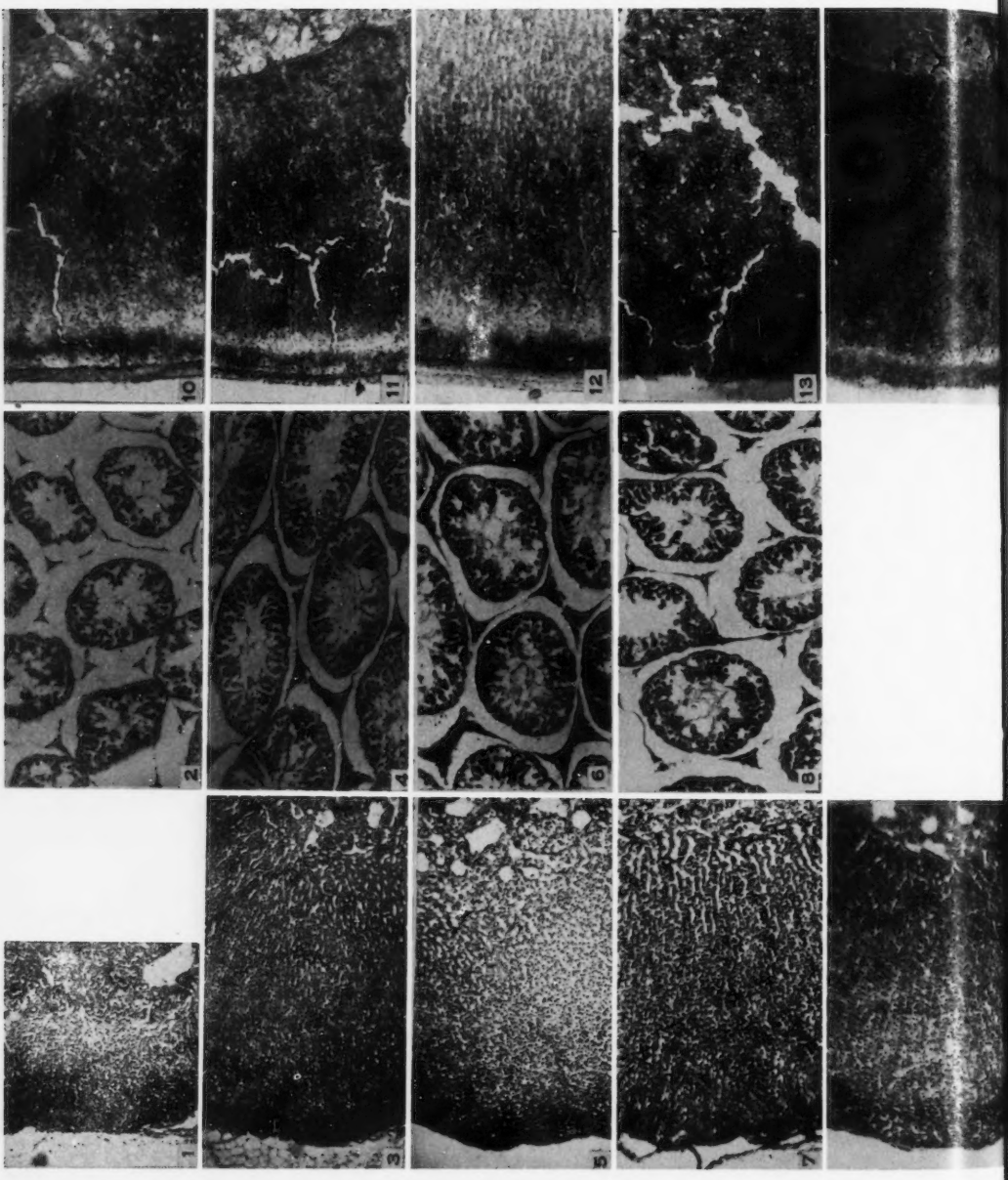
DISCUSSION

These observations lead us to believe that the adrenotropic action of pregnancy plasma was caused by CG, which stimulated the testes of the test animals to produce a steroid secretory product. The steroid in turn

TABLE 2. ASSAY FOR ACTH ACTIVITY*

Material	Dose/rat	Ascorbic acid (mg/100 gms. adrenal tissue)		
		Before ACTH	After ACTH	Net change
ACTH	0.011 I.U.	496	271	−225
ACTH	0.0011 I.U.	475	367	−108
Sheep luteinizing hormone	2 mg Armour standard	496	441	− 55
Equine gonadotropin	600 I.U.	479	460	− 19
Chorionic gonadotropin	1000 I.U.	414	442	+ 28
Maternal plasma pool 4	0.6 cc.	401	439	+ 38

* None of the materials tested had as much as 0.0011 i.u. ACTH activity.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acted on the adrenals of the hypophysectomized rats to partially maintain their weight. Three types of studies are presented to afford evidence of this mechanism.

First, the adrenotropic action of pregnancy plasma was imitiated by CG and other gonadotropins with luteinizing hormone activity. Blood bank plasma pools which contained no CG activity had no adrenotropic action. When CG was added to the inert bank plasma in amounts comparable to those in pregnancy plasma, the preparation reproduced the adrenotropic action of maternal plasma. The minimum dose of each necessary to produce the adrenotropic response was the same in terms of CG activity, and the maximimum response to higher doses of each was the same. Both produced identical histological effects in the rat adrenals and maintained the interstitial cells of the rat testes. The adrenotropic and histological effects of maternal plasma and of bank plasma with added CG were reproduced by sheep pituitary LH and by equine gonadotropin. This parallelism

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FIGS. 1, 3, 5, 7, 9 adrenals stained with H&E (×35). Figs. 2, 4, 6, 8 testes stained with H&E (×35). Figs. 10–14 adrenals stained with Sudan III (×35).

FIG. 1. Adrenal from a hypophysectomized rat which received no therapy. The cortex is thin.

FIG. 2. Testis from a hypophysectomized rat which received no therapy. The interstitial cells are atrophic.

FIG. 3. Adrenal from a hypophysectomized rat treated with maternal plasma. The cortex is thicker than in the untreated animal.

FIG. 4. Testis from a hypophysectomized rat treated with maternal plasma. The interstitial cells are not atrophic.

FIG. 5. Adrenal from a hypophysectomized rat treated with chorionic gonadotropin. The cortex is thicker than in the untreated animal.

FIG. 6. Testis from a hypophysectomized rat treated with chorionic gonadotropin. The interstitial cells are not atrophic.

FIG. 7. Adrenal from a hypophysectomized rat treated with ACTH. The cortex is thicker than in the untreated animal.

FIG. 8. Testis from a hypophysectomized rat treated with ACTH. The interstitial cells are atrophic.

FIG. 9. Adrenal from a hypophysectomized caste rat treated with testosterone propionate. The cortex is thicker than in the untreated animal and resembles those seen after treatment with maternal palsma, chorionic gonadotropin, and ACTH.

FIG. 10. Adrenal from a hypophysectomized rat which received no therapy. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 11. Adrenal from a hypophysectomized rat which received maternal plasma. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 12. Adrenal from a hypophysectomized rat which received chorionic gonadotropin. A zone devoid of fat-staining material is present in the fascicular zone.

FIG. 13. Adrenal from a hypophysectomized rat which received ACTH. There is a fairly uniform distribution of fat in the cortex and no fat-free zone is present.

FIG. 14. Adrenal from a hypophysectomized, castrate rat which received testosterone propionate. A zone devoid of fat-staining material is present in the fascicular zone.

TABLE 3

	Adrenal weight (mg.)	
	Mean	Std. error of mean
A. *Female hypophysectomized rats*		
Control (Bank Plasma Pool 2)	17.1	0.3
Chorionic Gonadotropin (25 I.U./cc.)	17.6	0.8
Maternal Plasma Pool 5	18.8	0.9
B. *Male castrate hypophysectomized rats*		
Control (Bank Plasma Pool 2)	19.1	0.6
Chorionic Gonadotropin (25 I.U./cc.)	18.3	1.0
Maternal Plasma Pool 5	19.0	0.5
C. *Male castrate hypophysectomized rats**		
Control (sesame oil)	12.3	0.6
Testosterone propionate 2 mg./day	20.5	1.6
D. *Intact male rats**		
Control	42.4	1.4
CG (25 I.U./cc.)	40.0	0.9

A and B: Neither chorionic gonadotropin (CG) nor maternal plasma caused a change in adrenal weight in hypophysectomized female rats or in hypophysectomized castrate male rats. Control values in these animals are higher than in male non-castrate hypophysectomized rats and reflect higher adrenal weights at the beginning of treatment.

C: Increase in adrenal weight in hypophysectomized, gonadectomized male rats given testosterone propionate in sesame oil ($P < 0.01$).

D: CG had no effect on adrenal weights in intact male rats.

Analysis of variance revealed no significant differences within groups A, B, and D.

* From Charles River Breeding Laboratories, Brookline, Massachusetts.

of action between maternal plasma, CG, LH, and equine gonadotropin suggested that the active principle was luteinizing hormone activity, a property they shared in common as evidenced by the ovarian hyperemia response to each.

Second, ACTH also maintained adrenal weight in hypophysectomized rats, but ACTH activity in the various test materials cannot account for their effectiveness in adrenal weight maintenance. ACTH assay revealed that in the test doses employed, neither maternal plasma, CG, LH, nor equine gonadotropin contained sufficient ACTH for adrenal weight maintenance. For example 2 mg. of LH contained less than 0.0011 I.U. ACTH activity. The LH given in the adrenal weight maintenance experiments contained 1.0 mg./cc., or less than 0.00055 I.U./cc. ACTH activity. This was $<1/150$ the dose of ACTH required to produce an equivalent response (0.083 I.U./cc.; table 1B). Similar calculations revealed factors of $<1/45$ for maternal plasma, $<1/300$ for equine gonadotropin, and $<1/25{,}000$ for CG. The factors for maternal plasma and CG are conservative estimates; 0.0011 I.U. ACTH activity over-estimates their possible ACTH content, since neither showed any activity at all in the assay. In addition, histological evidence indicated the lack of ACTH activity in the test materials. In the absence of ACTH, a characteristic sudan free zone appears in the outer part of the fascicular zone (4) (Fig. 10). Adrenotropic doses of maternal plasma, CG, LH, and testosterone propionate failed to eliminate

this zone in the hypophysectomized rats, but a similarly adrenotropic dose of ACTH caused it to disappear.

Third, maternal plasma and the gonadotropins which imitated its action all maintained the interstitial cells of the testis, suggesting that the secretory product of these cells might be the adrenotropin. Further experiments showed that this was the case. The presence of the testis was essential for either maternal plasma or CG to show an adrenotropic action. Neither of these materials prevented adrenal involution in either hypophysectomized female rats or hypophysectomized and gonadectomized male rats. However, testosterone propionate administered to hypophysectomized and gonadectomized male rats was effective in maintaining adrenal weight in the same way as CG or maternal plasma in hypophysectomized male rats. This effect of testosterone on the adrenal of hypophysectomized rats is confirmed by previous work of other investigators (5, 6, 7) whose studies demonstrated partial weight maintenance of the adrenal in hypophysectomized male rats, using doses of 1.25 to 2.5 mg. testosterone propionate. Our own experience and that of others (7) indicates that lower doses are less effective and that the effect disappears when the dose of testosterone is reduced to about 0.2 to 0.5 mg. per day.

Maintenance of adrenal weight by steroid therapy has also been reported in rats with adrenal atrophy induced by cortisone administration instead of by hypophysectomy (8, 9, 10). In these animals, a large number of C-19 and methylated C-20 steroids were shown to be effective to varying degrees in the prevention of adrenal weight loss. The most effective steroids were androgenic, although their effectiveness bore no constant relationship to androgenicity, chemical structure, or other recognized factor. These findings appear to support our interpretation of our own results in hypophysectomized rats, in which we believe that adrenal weight was partially maintained by steroids secreted by the rat testes under gonadotropic-stimulation.

We first suspected that CG might cause the partial adrenal weight maintenance in hypophysectomized rats because of previous evidence that CG was related to the striking adrenal hypertrophy in the human fetus (11, 12). This evidence remains attractive, but the mechanism by which we believe CG partially maintains the adrenal of hypophysectomized male rats does not appear to be directly applicable to the problem of human fetal adrenal hypertrophy for several reasons. First, the differences in adrenal weight which we have demonstrated represent only a partial prevention of involution. The adrenal weight of 40-day old intact male rats was 37.5 $\pm$1.8 mg. At the end of the 8-day experiment, adrenal weights were of the order of 15 mg. for controls and 20 mg. for CG treated rats. Extensive involution had therefore occurred in both groups and had been only partially prevented by CG therapy. The human fetal adrenal, on the other hand, is greatly enlarged, being proportionately 10 to 20 times heavier than the

adult gland (13). Second, CG had no effect on adrenal weights in non-hypophysectomized rats. In the human fetus the pituitary appears to be essential for the adrenal hypertrophy to occur; anencephalic infants, which have severely defective pituitaries, usually do not show fetal adrenal enlargement. Third, chorionic gonadotropin is found in the fetus only in low titers relative to those in the mother (14), and we have found (unpublished observations) that neither fetal urine nor fetal plasma is adrenotropic in the hypophysectomized male rat. We believe, therefore, that if chorionic gonadotropin is related to human fetal adrenal development, as is suggested by other experiments, factors in addition to those demonstrated in these studies must be involved.

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CHANGES IN THE GOLGI APPARATUS OF THE ISLETS OF LANGERHANS IN THE RAT FOLLOWING GLUCOSE AND INSULIN ADMINISTRATION[1]

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ABSTRACT

The appearance of the Golgi apparatus of the beta cells of the islets of Langerhans of the rat was studied with a view to finding a better histological criterion of activity than those now available. The Golgi apparatus of the beta cell is clearly distinguishable from that of the alpha cell, since the former is much larger and more elaborate, while the latter is very small and compact. Maintenance of elevated blood glucose levels for 4 hours resulted in an increase in the size and intricacy of the beta cell Golgi apparatus, while at 12 hours the change was even more marked. Attention was then turned to the alpha cells to see if maintenance of hypoglycemia, said to be a stimulus to glucagon release, would produce alterations of their Golgi bodies. Insulin was infused continuously intravenously, resulting in blood glucose levels of 15–30 mg.% over at least a 4-hour period. In spite of the marked hypoglycemia, no changes in the Golgi apparatus of the alpha cells was observed, although the beta cell Golgi apparatus showed beginning involution.

A GROWING body of evidence indicates that the blood glucose level is one of the most important factors influencing the rate of insulin production. This relationship was first demonstrated by Banting and Gairns (1) and Houssay, *et al.* (2), while in a more extensive study, Zunz and LaBarre (3), using cross-circulation experiments, showed that production of hyperglycemia in the donor dog resulted in a decline in the blood glucose level of the recipient. Local infusions of glucose into the pancreas have been demonstrated by Kosaka (4) and Brown *et al.* (5) to result in hypoglycemia; the latter authors have also observed islet hyperplasia, degranulation and beginning hydropic degeneration in the part of the pancreas infused with glucose. The most direct piece of evidence was provided by Anderson and Long (6), who found that elevation of the glucose level in the blood perfusing the isolated rat pancreas resulted in an increase in the insulin content of the blood.

Like the cells composing most glandular tissue, the beta cells of the

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islets of Langerhans show alterations in their morphology under conditions which may be expected to increase their activity. Prolonged hyperglycemia in cats and dogs (7, 8, 9, 10) results in degranulation and hydropic degeneration, while the usual response in the guinea pig (11, 12, 13) and the rat (14, 15) consists only of degranulation which is followed by islet hyperplasia if the elevation in blood glucose is sufficiently prolonged.

While the beta cells usually respond acutely to a stimulus by degranulating, nevertheless Nerenberg (16) has shown that this is not always the case, since, in his experiments, fasting reduced the granule content and feeding of carbohydrate restored it. Therefore, the granule content is an unreliable criterion of activity in these cells. Indeed, as has been clearly pointed out many times, the degree of granulation at any time depends upon the balance between production and release. Since there are no good criteria of acute changes in beta cell activity, the first group of experiments outlined below was undertaken in an effort to discover other more reliable morphological changes which might be used to assess the degree of beta cell activity and which would occur sufficiently rapidly to be useful in acute studies. The Golgi apparatus seemed to be a likely candidate for such a role. While the argument concerning the actual nature of the Golgi apparatus is still in progress, it is nevertheless generally recognized that, when glandular tissue shows increased secretory activity, the Golgi apparatus in each cell becomes larger and more elaborate.

Studies of the Golgi apparatus in the islets of Langerhans have been curiously few. Ludford and Cramer (17) described hypertrophy of the Golgi bodies in the islets during pregnancy, elevation of body temperature, and after operative procedures. Vazquez-Lopez (18), although unable to impregnate the Golgi apparatus in the normal islets, described what he termed an hypertrophied Golgi apparatus in the islets of estrogen-treated rats. In neither stuy was any differentiation made between the Golgi apparatus of alpha and beta cells.

While it is quite clear that the beta cells produce insulin and that an elevation in blood glucose leads to an increase in their activity, the situation with respect to glucagon is not as well worked out. Until recently, there has been controversy concerning the cell of origin of this hormone. Recently, however, deDuve (19) reported that complete destruction of the alpha cells by high doses of cobalt is associated with the absence of extractable glucagon. In addition, Bencosme and Liepa (20) and Bencosme, Liepa and Lazarus (21) have found that the uncinate process of the dog pancreas, a region almost devoid of alpha cells, is also almost devoid of glucagon. In view of these findings, the conclusion that the alpha cells produce glucagon is almost inescapable. However, the factors leading to its release are less well defined. Foa *et al.* (22), using cross-circulation experiments in which the pancreaticoduodenal vein of the donor dog drained into the portal circulation of the recipient, found that hypoglycemia in

the donor dog resulted in hyperglycemia in the recipient, a finding which suggests that hypoglycemia is at least one of the ways by which glucagon release may be caused. It would therefore be anticipated that a prolonged depression of blood sugar level would result in morphological changes in the alpha cells due to their sudden increase in activity, and accordingly the second group of experiments was carried out as described below.

METHODS

Rats of the Long-Evans strain, 60–90 days of age and weighing 200–300 gm., were employed, female rats being used when glucose was infused and male rates when insulin was infused. All animals were fed a stock diet *ad libitum*. In some rats, diabetes was produced by the intravenous injection of 50 mg. per kg. of body weight of recrystallized alloxan following a fast of 72 hours. For the next 6 days, these animals were treated with 4 units per day of crystalline zinc insulin subcutaneously. At least 30 days then elapsed before the animals were used.

In the first group of experiments, glucose was infused continuously through a polyvinyl catheter in the tail vein. In the experiments of 4 hours duration or less, the animals were anesthetized with nembutal throughout, but in the 8 and 12 hour studies, the animals were anesthetized with ether during the placing of the catheter in the tail vein, and then allowed to recover. These animals were kept in restraining cages for the duration of the experiment. In the shorter experiments, the animals were first given a priming dose of 1 cc. of 25% glucose in 0.2% pyrogen-free NaCl intravenously, following which they were connected to a constant injection pump which delivered 5.3 cc. of 25% glucose in 0.2% NaCl per hour. In the 8 and 12 hour experiments, no priming dose was given and the glucose was infused as a 10% solution in 0.5% NaCl at a rate of around 15 cc. per hour. NaCl was included in the infusion to avoid depletion of body sodium and chloride as a result of the marked diuresis. At the end of each experiment, the infusion was stopped, the animals were anesthetized, and after a lapse of several minutes, blood was drawn from the vena cava for the measurement of blood sugar and the pancreas was removed and fixed for histological study.

In the second group of experiments, insulin was infused into both normal and alloxan-diabetic animals. Both groups of animals were infused with crystalline zinc insulin intravenously at a rate of 0.44 unit per hour (Lilly "Isletin" diluted with 0.9 pyrogen-free NaCl), but in addition, the alloxan-diabetic animals were given 2–3 units of insulin intraperitoneally two hours before the beginning of the experiment in order to bring the blood sugar levels down to roughly normal levels. As in the short glucose infusions, the animals were under continuous nembutal anesthesia from the time that the infusions were begun. Blood was drawn from the inferior vena cava every half or three-quarters of an hour for the determination of glucose. At the end of the experiment, the pancreas was removed and fixed.

Glucose was measured by the Nelson modification of the Somogyi method (23). Part of the pancreas was fixed in Bouin's fluid, embedded in paraffin, sectioned at 4 μ and stained with the Gomori aldehyde fuchsin following preliminary oxidation with 0.3% $KMnO_4$ in 0.3% H_2SO_4 for 1 minute with subsequent bleaching in 3% sodium bisulfite. A brilliant stain of the beta cell granules with almost no background staining was obtained in 4 minutes. After rinsing in 95% alcohol, the slides were stained for about 5 minutes in chrome hematoxylin (24), differentiated briefly in 1% HCl in 70% alcohol, washed in tap water for 5 minutes and counterstained for 20 minutes in the following mixture: 0.25 gm. chromotrope 2R, 0.1 gm. light green, 0.7 gm. phosphotungstic acid, 20 cc. glycerol, 80 cc. water, 1 cc. glacial acetic acid. Slides were rinsed briefly in 0.1%

acetic acid, dehydrated and mounted. The alpha cell granules were deep orange, the beta cell granules purple and the cytoplasm of both cells green. For demonstration of the Golgi apparatus, a second part of the pancreas was cut into small pieces and fixed for 24 hours in Champy-Kull fixative, washed in running water for 24 hours, then treated with 2% osmic acid at 40° C for 12 hours and at 35° C for 8 days. The tissues were washed for 24 hours in running water, dehydrated, embedded in paraffin, and sectioned at 4 μ. Sections too heavily impregnated with osmic acid were bleached in 0.125% $KMnO_4$ followed by 3% sodium bisulfite. Some preparations of pancreas impregnated with osmic acid were counterstained with Mallory-Azan for the differentiation of cell types.

RESULTS

The Normal Islet

The appearance of the islets of Langerhans in the normal rat following application of specific granule stains has been amply described. In all normal controls, the usual preponderance of beta cells over alpha cells was observed together with a general grouping of beta cells centrally and alpha cells around the periphery. While the beta cells of smaller islets were usually filled with granules, the largest islets contained numerous cells with granules confined to the borders of the cells.

The appearance of the islets after impregnation of the Golgi apparatus requires more extensive comment, inasmuch as so little work appears where this technique was used. When islets of normal animals were examined (Fig. 1), it was apparent at once that there were two types of Golgi apparatus and it was necessary to determine whether the cells possessing the two different Golgi types could be identified with the alpha and beta cells. Most of the cells in the islet had a relatively large, moderately elaborate Golgi body, which was located close to the nucleus and formed a sort of cap over it. The cells containing this type of Golgi apparatus were fairly large and formed the central part of the islet, thus correspondong in position and size, at least, to the beta cells. In addition, their cytoplasm contained granular material which had a tendency to reduce osmic acid, thus lending a greyish tint to the cytoplasm. Located at the edges of the islets, frequently in a cap at one end, were smaller cells, all of which had a clear cytoplasm and which had a very small, exceedingly simple Golgi apparatus near one pole of the nucleus. These cells, then, corresponded in their position and size to the alpha cells. Since it proved impossible to counterstain these preparations with either aldehyde fuchsin or chrome hematoxylin, they were finally counterstained with Mallory-Azan, with the result that the granules of the small cells with the small Golgi bodies stained with azocarmine, while the others did not, thus indicating that probably the former were the alpha cells. In an effort to put this distinction on a still firmer footing, the appearance of the islets of alloxan-diabetic rats was studied following impregnation of the Golgi apparatus (Fig. 2). It is well-known that rats severely diabetic as a result of alloxan treatment show almost complete disappearance of the beta cells, leaving islets composed almost exclusively of alpha cells. The few beta cells left are

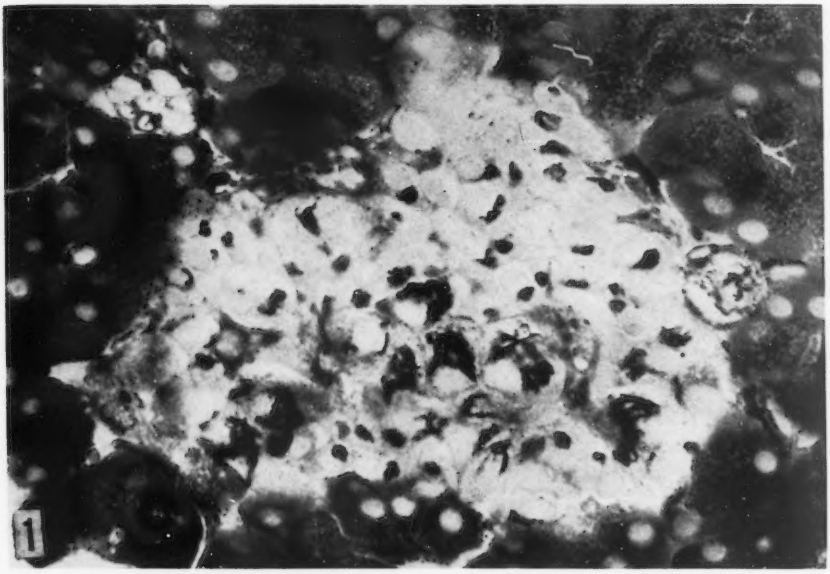

FIG. 1. Golgi preparation of islet from a normal rat. Note centrally placed large cells with large Golgi bodies and dark cytoplasm and peripherally placed small cells with small, very simple Golgi bodies. ×917.

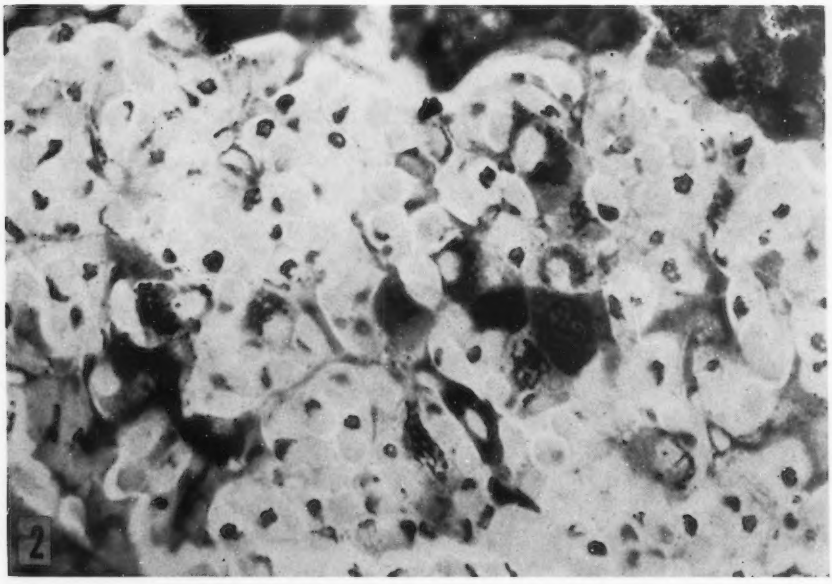

FIG. 2. Golgi preparation of islet from an untreated alloxan-diabetic rat. Note almost complete disappearance of beta cells, leaving the islet composed almost entirely of alpha cells with small Golgi bodies and clear cytoplasm. ×917.

usually considerably enlarged. In this series, these changes were confirmed in material fixed in Bouin's fluid and stained with aldehyde fuchsin. In the Golgi preparations from these animals, almost the entire islet was composed of the small cells with clear cytoplasm and small Golgi bodies. Occasionally, there were seen one or two quite large cells with greenish cytoplasm and an enormously large and elaborate Golgi apparatus. The latter cells would logically seem to be representatives of the few beta cells left, which had greatly hypertrophied as a result of the hyperglycemia, while the small cells appeared to be identical with the alpha cells.

Group I: Glucose Infusion

Sixteen experiments were carried out in which the duration of the infusion varied from $1\frac{1}{2}$ to 12 hours. The infused animals were found to have blood sugar levels ranging from 327 to 1310 mg.% at the end of the experiments.

Following the infusion of glucose, the beta cell granulation was greatly reduced, a finding which has been repeatedly described by others. This degranulation was almost completely confined to the medium- and large-sized islets, with the small islets appearing not to participate in the response. In all experiments the degree of degranulation was about the same regardless of the duration of the hyperglycemia. The granulation of the alpha cells appeared to be unchanged.

In osmic acid preparations from these animals, the Golgi bodies of the beta cells had become somewhat larger and considerably more elaborate (Figs. 3, 4). This process was discernable in the animals infused for only $1\frac{1}{2}$ hours, was well-established at 4 hours and was quite marked at 12 hours. The Golgi apparatus of the alpha cells did not appear to change in any way.

Group II: Insulin Infusion

Four normal animals were infused with insulin intravenously at a rate of 1.3–1.8 units kg./hr. The blood sugar declined promptly, reaching 40 mg.% or below within an hour, and was maintained at this level for an interval which varied from $1\frac{1}{2}$–3 hours. This persistent hypoglycemia produced no change in the granule content or other appearance of either alpha or beta cells in the material stained with aldehyde fuchsin. There was a slight shrinkage of the Golgi bodies of the beta cells, but in the alpha cells they were unchanged.

Inasmuch as the number of alpha cells compared to beta cells was small in the normal animal, making their study more difficult, 5 alloxan-diabetic rats were infused with insulin in the same way and at the same rate as the normal rats, except that 2 nours before the beginning of the experiment, they were given 3 units of regular insulin to bring their blood sugars down into the normal range. For at least 4 hours, their blood sugars were between

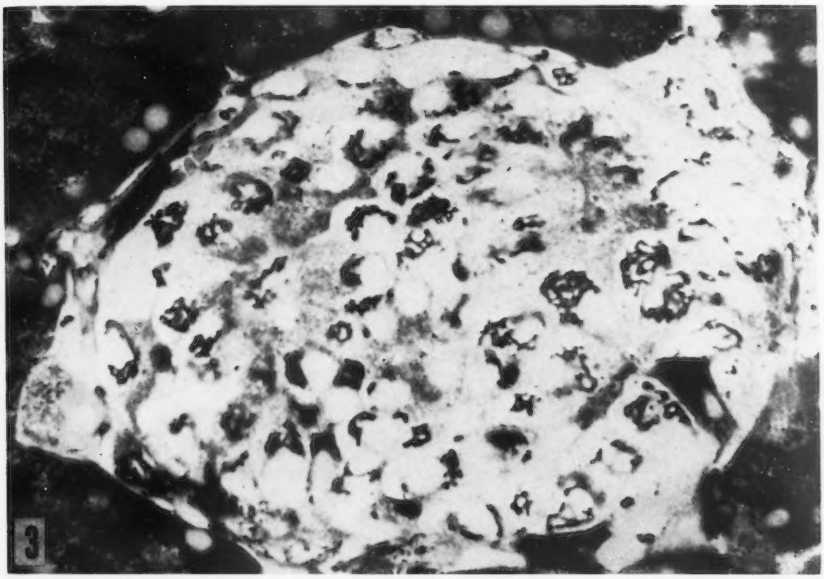

FIG. 3. Golgi preparation of islet from a normal control rat. Islet is composed almost entirely of beta cells. A few cells show elaborate Golgi bodies, but in general they are small and simple. ×917.

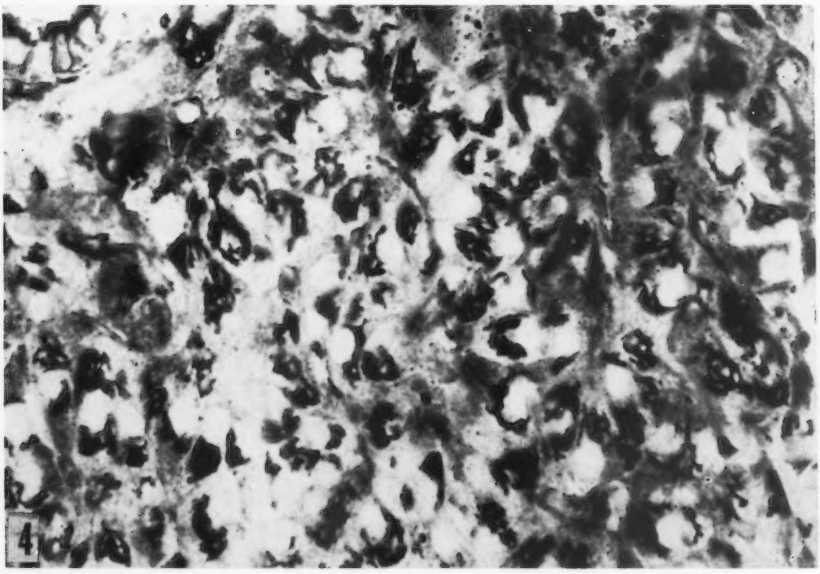

FIG. 4. Golgi preparation of islet from a normal rat infused with glucose for 4 hours. Note generalized increase in complexity of Golgi bodies in all cells. ×917.

15 and 30 mg.%. With the aldehyde fuchsin stain, the untreated alloxan-diabetic controls showed the usual masses of well-granulated alpha cells with an occasional degranulated beta cell. The animals infused with insulin showed no changes in the granule content of the alpha cells, nor did the few beta cells change in appearance. The appearance of the islets of untreated diabetic rats in Golgi preparations has been described above (Fig. 2). In the Golgi preparations from diabetic rats given insulin, none of the alpha cells showed any enlargement or elaboration of the Golgi apparatus (Fig. 5).

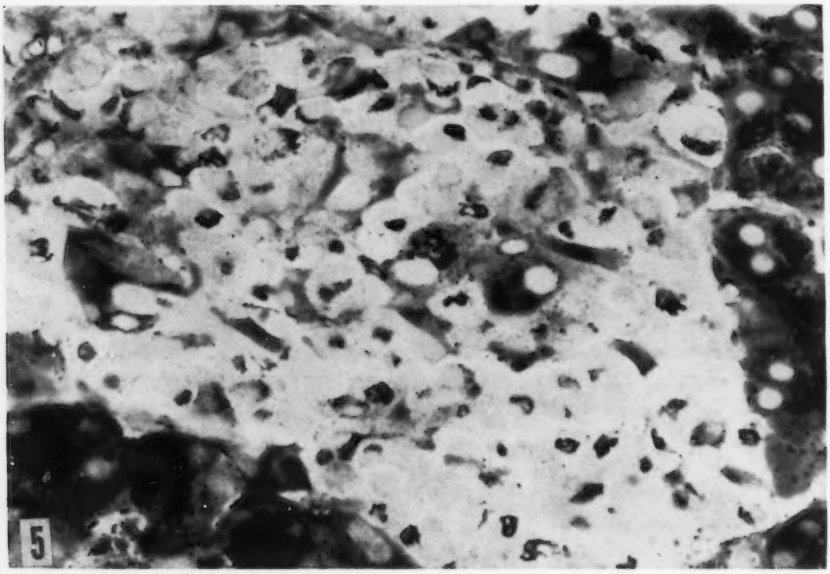

FIG. 5. Golgi preparation of islet from an alloxan-diabetic rat infused with insulin for 4 hours. Islet is composed almost entirely of alpha cells whose Golgi bodies are indistinguishable from those in Fig. 2. ×917.

DISCUSSION

Since the appearance of the Golgi apparatus changes fairly rapidly when the beta cells are presented with a stimulus to insulin production in the form of hyperglycemia, we have at hand a means of assessing the level of activity of these cells which is probably better than that afforded by observation of granule content alone, inasmuch as the amount of granular material undoubtedly represents a balance between production and release. If, as has been shown here, the Golgi apparatus of the beta cells changes its appearance within 4 hours after the beginning of an adequate stimulus to these cells, one might perhaps expect that the Golgi apparatus

of the alpha cells would show recognizable alterations within roughly the same time interval following the beginning of hypoglycemia, which should be an adequate stimulus to their activity. The finding that the alpha cells did not change in any way is puzzling. Of course, several assumptions were made in this experiment. The first was that hypoglycemia is in fact a stimulus to the production of glucagon in the rat, as it has been reported to be in the dog (22). A second assumption was that glucagon is rapidly released when an adequate stimulus is provided. However, the available evidence does indicate that glucagon is released within minutes after the production of hypoglycemia (22). A third assumption was that the morphology of the alpha cells would change when they are stimulated in about the same length of time required by the beta cells to change. A fourth assumption is that the alpha cells are indeed the site of glucagon production, and, if it were not for the strong evidence already cited, one would be tempted to doubt that the alpha cells produce glucagon, since, in this experiment and in many others, their morphology remained unchanged under a variety of conditions said to produce release of glucagon. For example, Fodden and Read (25) reported increased blood levels of glucagon after 12 days of GH treatment, and Bornstein, Reid and Young (26) found a hyperglycemic effect of pancreatic venous blood of cats treated with GH for 5 days and in rats treated for 24 hours. Also Foa *et al.* (27) reported that single injections of GH resulted in the release of a substance from the pancreas which caused hyperglycemia in cross-circulation experiments. In spite of these findings, the reports of the effects both of hypophysectomy and of GH administration on the morphology of the alpha cells have been variable. Although most workers have found no changes after hypophysectomy, Ferner and Tonutti reported atrophy of the alpha cells (28). The reports of Ferner have been criticized by Volk, Goldner and Frank-Crowley (29) because of the capricious nature of the Gros-Schultze silver method used to demonstrate the alpha cells. The latter authors, using more reliable techniques, were unable to find any differences after hypophysectomy. The effect of GH administration has also been highly variable. The alpha cells have been reported as unchanged after GH treatment (30), while on the other hand others have observed increased mitotic activity (31), or some increase in size, but only when corticotropin was simultaneously administered (32). The effect of the other known stimulus for glucagon release, namely hypoglycemia, has not been investigated in chronic experiments with the exception of one study (33) in which the alpha:beta cell ratio was reported as increased following chronic insulin administration, but unfortunately, no consideration was given to the possibility that this might have been due to a decrease in number of the beta cells. If the alpha cells do produce glucagon under the variety of conditions just outlined, then they are remarkable in their ability to do so with a minimum of morphological change.

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A METHOD FOR THE BIOLOGICAL ASSAY OF PHOSPHATURIC ACTIVITY IN PARATHYROID EXTRACTS[1]

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ABSTRACT

A biological assay method has been developed for estimation of the phosphaturic activity of parathyroid extracts. It is based on the increased urinary excretion of inorganic phosphate by 2-month old male rats during the first 6 hours after parathyroidectomy. The extract to be tested is injected immediately after parathyroidectomy and just before beginning the collection of urine. Various factors of importance in the assay method were studied, including the use of parathyroidectomized rather than intact rats, the optimal time and the completeness of the urine collection, a stock diet rather than a low-calcium diet, and the dose-response curve. The most favorable conditions yielded a reasonably rapid and sensitive assay method with a mean index of precision (λ) of 0.30. Several applications of the method are described, in particular the estimation of the ratio of phosphaturic to calcium-mobilizing activity in parathyroid extracts.

A METHOD of biological assay for phosphaturic activity, for use in conjunction with a method (1, 2) already available for calcium-mobilizing activity, was needed to aid in the purification of parathyroid extract and to help settle the question whether a single hormone or two separate hormones are responsible for the two activities. This paper comprises a report of the experiments leading up to the development of such a bioassay method and descriptions of the recommended procedure and some of its applications.

MATERIALS AND METHODS

Male albino rats of uniform age obtained from the Holtzman Rat Co. were kept in pairs in suspended wire mesh cages in an air conditioned animal room with a controlled temperature of approximately 78° F. The stock diet was Purina laboratory chow. Analysis of a sample showed 0.94% calcium and 0.35% phosphorus. The low-calcium diet, a modification of the diet of Shaw (3), is described in Table 1. Parathyroidectomy was carried out under ether anesthesia by cauterization with a hot wire.[2] For collection of urine each rat was placed in an individual metal metabolism cage with drinking water available but no food. The spontaneously voided urine was collected in glass jars con-

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[2] Details of the technique of parathyroidectomy will be supplied on request.

TABLE 1. LOW CALCIUM DIET

	gm./kg.
Sucrose	684
Casein with added vitamins[a]	240
Salt mixture[b]	26
Corn oil with added vitamins[c]	50

[a] *Casein with added vitamins.*
To each 100 gm. of casein (De-Vitaminized Casein from Sheffield Chemical Co., Norwich, N. Y.) add:

	mg.
Thiamine hydrochloride	1.46
Riboflavin	1.46
Pyridoxine hydrochloride	1.46
Nicotinic acid	10.4
Calcium pantothenate	8.3
Choline chloride	417.0
Inositol	4.7
Para-aminobenzoic acid	126.0

[b] *Salt mixture*
Mix the following salts:

	gm.
K_2HPO_4	645
$NaH_2PO_4 \cdot H_2O$	126
$MgSO_4$	100
NaCl	282
Ferric citrate $\cdot H_2O$	55
KI	1.6
$MnSO_4 \cdot H_2O$	9.0
$ZnCl_2$	1.0
$CuSO_4 \cdot 5H_2O$	0.6

[c] *Corn oil with added vitamins*
To each 100 gm. of corn oil add:

β-Carotene	22 mg.
Irradiated ergosterol	6000 I.U.
2-Methyl-1,4-naphthoquinone	12 mg.
α-Tocopherol	100 mg.

taining 1 ml. of 2N hydrochloric acid. At the end of the collection period, the funnels of the cages were washed down with a stream of distilled water, and the combined urine and washings were diluted to 100 ml. with distilled water. The total urinary inorganic phosphate for the period was determined by the method of Fiske and Subbarow (4). The crude parathyroid extracts were prepared by extracting ground frozen bovine parathyroid tissue[3] with 10 times the weight of 0.1N hydrochloric acid for 15 minutes at the temperature of a boiling water bath. The extracts were standardized against Parathyroid Extract U.S.P.[3] by the assay method of Munson *et al.* (1, 2) for calcium-mobilizing activity. Two extracts were used in this study. One extract, P20, contained approximately 28 U./ml., and the other, P22, contained 36 ± 8 U./ml.[4] The extracts contained a negligible amount of inorganic phosphate.

RESULTS

Comparison of Intact with Parathyroidectomized Rats and the Effect of the Duration of Collection of Urine. Male rats were maintained on the stock

[3] Generously donated by Eli Lilly and Co., through the courtesy of Dr. K. K. Chen.

[4] In this paper, a value following the ± sign without other designation denotes the standard error. A value followed by the abbreviation (s.d.) denotes the standard deviation.

diet for 7 days after receipt from the supplier. At the time of the experiment they were 46 days old and 120–150 gm. in weight. They were randomly divided into two groups, parathyroidectomized and intact, and each group was further divided into two subgroups, control and injected. The control rats received no treatment. The injected rats were given 3.0 ml. of parathyroid extract P20 subcutaneously per 100 gm. body weight. The parathyroidectomized rats were injected immediately following the operation. Urine was collected individually from each rat for 24 hours after the injection in three consecutive periods, 6, 6, and 12 hours, respectively.

The urinary excretion of inorganic phosphorus, in mg. per hour, for each period is shown in Figure 1. The untreated parathyroidectomized rats excreted phosphate at a much lower rate than the intact rats in all three periods. Injection of parathyroid extract was followed by a marked increase in urinary phosphate in both the intact and the parathyroidectomized rats, but for unknown reasons the increase was greater in the parathyroidectomized rats. The effect of the extract on urinary phosphate was greatest in the first 6 hours. The increase over the controls was considerably diminished in the second 6-hour period and was further diminished in

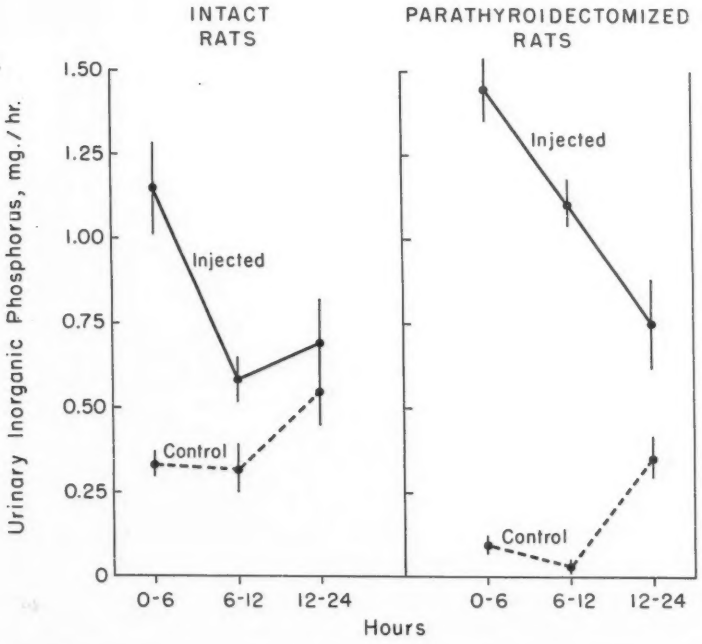

FIG. 1. Rate of urinary excretion of inorganic phosphorus by intact and parathyroidectomized rats. Parathyroid extract (84 units/100 gm.) was administered to the Injected rats; the Control rats were untreated. Each point (•) represents the mean of a group of 6 or 7 rats. The vertical line through each point represents the standard error.

the next 12 hours, although, at least in the parathyroidectomized rats, the difference between the injected and the control rats was still highly significant.

The increased rate of urinary excretion of phosphate by the untreated intact rats during the last 12 hours may indicate the beginning of a catabolic phase in the fasting animal. An additional factor contributing to the increase shown by the untreated parathyroidectomized rats during the same period may have been a marked rise in plasma inorganic phosphate resulting from the earlier retention of phosphate.

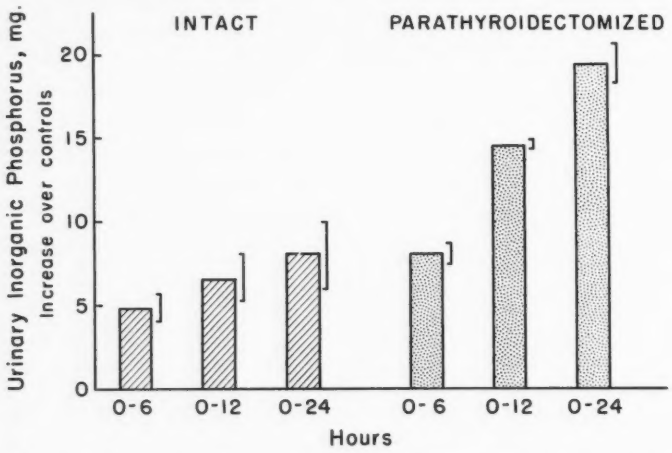

FIG. 2. Effect of parathyroid extract (84 units/100 gm.) on the cumulative urinary excretion of inorganic phosphorus by intact and parathyroidectomized rats. The height of each column represents the difference between the mean values for injected and control rats. There were 6 or 7 rats in each group. The brackets represent the respective standard errors.

The cumulative excretion of inorganic phosphorus by the injected rats for 6, 12, and 24 hours, after subtraction of the corresponding control value, is shown in Figure 2. Although the maximum increase in *rate* of phosphate excretion occurred during the first six hours, the *total effect* of the injection was greater when summated over the entire 24 hours, at least in the parathyroidectomized rats. The selection of a 6-hour period for collection of urine for the bioassay was therefore a matter of convenience rather than necessity, and it is acknowledged that a longer collection period might prove to be advantageous.

Standard Procedure for the Biological Assay Method. On the basis of the results of the preceding experiment a biological assay method was designed employing acutely parathyroidectomized male rats, eight to nine weeks of age. The rats were maintained on the stock diet for approximately one week after receipt from the supplier, and were then arranged in order of de-

creasing weight and assigned to treatment groups by formal randomization. They were next, in rapid succession, anesthetized with ether, parathyroidectomized, injected subcutaneously with parathyroid extract, and put into metabolism cages. The urine was collected for the following 6 hours. In the usual experimental design there were two dose levels of a suitable standard extract at a four-fold dose interval and as many unknowns as convenient, with 5 to 8 rats in each treatment group. The potency of an unknown was estimated in relation to the standard as described by Bliss (5) for parallel-line assays.

Results with the Standard Procedure. Eleven assays, using 232 rats, were conducted according to the standard precedure. The composite log dose-response curve of the 9 statistically valid assays is shown in Figure 3. In the remaining 2 assays the regression of the effect on the log dose of standard was not statistically significant. The mean index of precision (λ) of the 9 valid assays was $0.30 \pm .083$ (s.d.), and the minimum expected standard error of the estimated potency of an unknown when tested at 2 dose levels against 2 dose levels of standard, using 6 rats per group, was 33%.

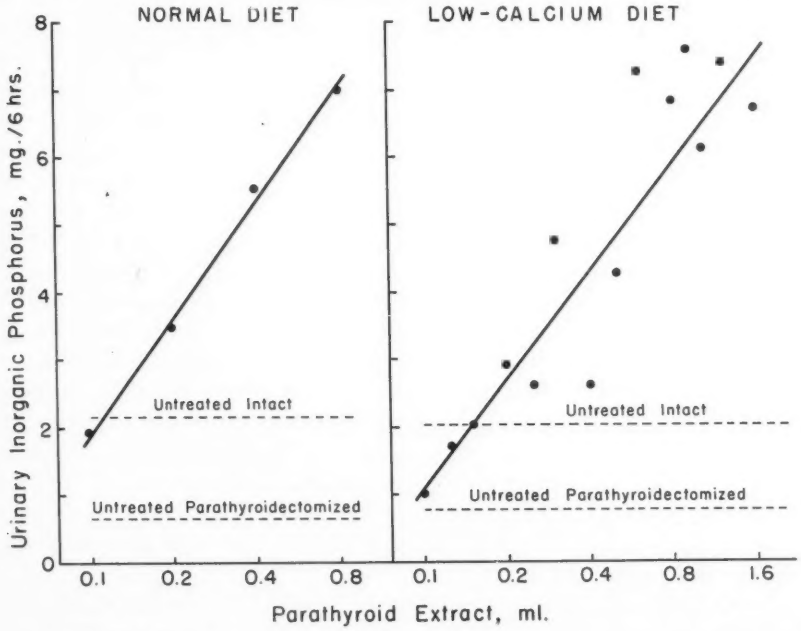

FIG. 3. Composite log dose-response curves for the biological assay methods for phosphaturic activity. Standard procedure (Normal Diet) on the left, modified procedure (Low-Calcium Diet) on the right. The horizontal dotted lines represent the average level of urinary excretion of inorganic phosphorus over a period of 6 hours for, respectively, intact and parathyroidectomized rats on the two diets. The standard errors of these control values ranged from 0.07 to 0.62 mg./6 hrs.

Results with a Modified Procedure. The biological assay method of Munson *et al.* (1, 2) for the calcium-mobilizing activity of parathyroid extract employs an experimental design identical with the standard assay procedure for phosphaturic activity except for the maintenance of the rats on a low-calcium diet for the four days immediately preceding the assay. With this modification, therefore, it is possible to conduct both assays on the same rats simultaneously. However, in a total of 36 simultaneous assays using 984 rats, the data on urinary phosphate provided only 22 valid assays. The composite log-dose response curve of the 22 valid assays is shown in Figure 3. The remaining 14 assays were invalid because of failure to achieve a statistically significant regression. The mean index of precision (λ) of the 22 valid assays by the modified procedure was 0.37 ± 0.15 (s.d.) as compared with 0.30 ± 0.08 (s.d.) for the standard procedure. Our preference for the standard procedure is based on both the possibly lower precision ($P < .2$) of the valid assays and the high proportion (39%) of invalid assays obtained with the modified procedure.

Completeness of Urine Collections. Although at the beginning of the urine collection period emptying of the bladder usually occurs during the anesthesia required for the operation, at the end of the 6-hour period the rats are removed from the metabolism cages without receiving any stimulus for micturition. Thus part of the observed variation between rats may have been due to incomplete collection of the urine. In one assay, by the modified procedure, the urine was analyzed for creatinine (6) as well as for phosphate. The assay statistics were computed in two ways, first, as usual, ignoring the values obtained for creatinine, and secondly, using the phosphorus to creatinine ratio. By the use of the phosphorus: creatinine ratio λ was apparently reduced, from 0.42 to 0.32. Although this change was not statistically significant, the ratio of the residual variances (F) was 2.14, with only 2.30 required for significance at the P.05 level. The results therefore suggest that incompleteness of urine collection may have contributed to the observed variation between rats and that the use of the phosphorus: creatinine ratio or deliberate manual emptying of the bladder might reduce the error of the assay.

Applications of the Assay Method for Phosphaturic Activity

1. Comparison of two parathyroid extracts for relative amounts of calcium-mobilizing and phosphaturic activity.

It was now possible, with the aid of the two biological assay methods, to compare the relative amounts of the two activities in various extracts. As reported previously (2, 7), our crude parathyroid extract, P22, was assayed twice for calcium-mobilizing activity and three times for phosphaturic activity. When the data were combined, P22 was found to contain $36 \pm 8\%$ of the calcium-mobilizing activity and $134 \pm 24\%$ of the phosphaturic activity of an equal volume of the commercial U.S.P. extract. The ratio of

phosphaturic to calcium-mobilizing activities was 3.6 ± 0.67 times as great in P22 as in the commercial extract.

2. Assay of spleen extract.

It has been suggested that because extracts of spleen and thymus were found to be phosphaturic in the dog that the phosphaturic activity of parathyroid extracts is not hormonal in nature (8). Ground, frozen bovine spleen was extracted with hot acid as described above for parathyroid extract. No phosphaturic activity could be detected in this spleen extract by our assay method in parathyroidectomized rats, and if any was present, it was less than $\frac{1}{16}$ that of crude parathyroid extract. There was also no detectable calcium-mobilizing activity in the spleen extract.

3. Assay of dialysates of parathyroid extract.

Various parathyroid extracts were subjected to equilibrium dialysis in cellulose tubing, $1\frac{3}{4}$ in. flat width, for 6 days at 4° C. and pH 2 (9). The original extract, the dialysand, and the dialysate were assayed for phosphaturic activity by the present method. A considerable proportion, approximately 20%, of the total phosphaturic activity of the extract was found in the dialysate. Calcium-mobilizing activity was also found in the dialysate, in contrast with the first report by Davies and Gordon (10) but in conformity with their later studies in which parathyroid extract was ultrafiltered through dialysis tubing (11). The ratio of the two activities in the dialysate was not significantly different from that in the dialysand or in the starting material.

DISCUSSION

The biological assay method for phosphaturic activity that we have described, although it falls somewhat short of the desired order of precision, appears to be more precise than alternative methods, recently reviewed by Greep and Kenny (12). Further possible improvements were noted in the Results section and are currently under investigation.

The modified assay procedure, described above, is conducted in conjunction with the assay for calcium-mobilizing activity, with the expenditure of little additional effort. Although the use of the modified procedure increases the chance of obtaining an invalid assay, it supplies data of at least a preliminary nature which, if sufficiently interesting, may be checked by a separate and more precise phosphaturic assay using rats maintained on a normal diet.

The specificity of the assay method for phosphaturic activity has not yet been studied extensively. For example, the importance of changes in renal hemodynamics during the collection of urine has not been assessed in relation to the effect on the tubular reabsorption of phosphate. Crawford *et al.* (6) state that ordinarily the contribution of increased glomerular filtration rate is of little importance in rats.

The suggestion by Stewart and Bowen (8) that the phosphaturic activity

of parathyroid extracts is an artifact also found in extracts of thymus and spleen was not supported in the present investigation in which bovine spleen extract was found to contain no detectable phosphaturic activity in rats. The thoroughly documented observations that parathyroidectomy is followed by retention of phosphate and that administration of parathyroid extract results in an increased urinary excretion of phosphate certainly favor the existence of a phosphaturic parathyroid hormone. It is possible, however, that not all of the phosphaturic activity of parathyroid extracts is hormonal in nature.

The factor of 3.6 between the ratios of phosphaturic to calcium mobilizing activity in a crude parathyroid extract and in a commercial U.S.P. preparation (2, 7) has been confirmed by Davies *et al.* (13). The possible significance of this discrepancy in ratios for current experience (14, 15, 16, 17, 18) with the Ellsworth-Howard test (19) has been discussed elsewhere (2, 12). Differences in the ratios of the two activities in different extracts would indeed not be surprising if the parathyroid contained two hormones. Proof of this interesting hypothesis will require chemical separation and purification of two hormones. One (20) of three recent reports on purified parathyroid preparations states that both phosphaturic and calcium-mobilizing activities were present. There are other possible explanations of the discrepancy in ratios. First, the crude extract may contain nonhormonal phosphaturic material. Secondly, as noted by Rasmussen and Westall (21), the difference between the two extracts may be pharamacological in nature, since the assay for calcium-mobilizing activity is based on a measurement at a certain instant of time while the assay for phosphaturic activity is based on a cumulative response. Whatever the cause of the difference in assay ratios, a real difference in hormone content, the variable presence of artifact, or a difference in duration of action, caution should be exercised in comparing the phosphaturic activities determined by different assay methods. The definition of a unit of phosphaturic activity in terms of a standard assay method or, preferably, the establishment of a reference standard would tend to minimize the problem.

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THE EFFECTS OF COBALT INJECTIONS ON TOTAL CIRCULATING RED CELL VOLUME AND BONE MARROW CYTOLOGY IN NORMAL AND ADRENALECTOMIZED DOGS

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ABSTRACT

Adrenalectomized and intact female dogs were injected with cobalt alone for 30 days. Adrenalectomized dogs were injected for an additional period with a combination of cobalt and hydrocortisone. At 10-day intervals measurements were made of the hematocrit, hemoglobin, red cell counts and body weight. Total circulating red cell volume (P^{32} method) was measured at the end of and at various intervals during the course of the experiment. Femoral bone marrow myelograms and total nucleated cellularity were determined on the day following the last injection in each dog.

Intact dogs responded to cobalt injections with a significant increase in hematocrit, red cell count, hemoglobin, red cell volume and erythroid precursors within femoral bone marrows, while the blood picture and bone marrow cytology of adrenalectomized dogs injected with cobalt were not significantly different from those of desoxycorticosterone-maintained adrenalectomized dogs. Treatment of adrenalectomized dogs with a combination of cobalt and hydrocortisone tended to suppress the myeloid hyperplasia seen in the bone marrows of DCA-maintained adrenalectomized dogs. These data are interpreted to mean that an adequate supply of adrenocortical hormones is necessary for the hematopoietic response to cobalt.

WE HAVE reported previously that the adrenalectomized rat does not respond to cobalt injections with a characteristic polycythemia, as do sham operated controls, until they develop accessory adrenals (1). Stewart and Rogoff (2) reported that only one of 150 adrenalectomized dogs was found with an accessory adrenal at autopsy. Therefore, the dog appears to be a more reliable test animal than the rat for evaluating the effects of adrenalectomy on cobalt polycythemia over a prolonged interval provided adequate maintenance therapy is instituted immediately after operation.

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In view of these facts and to further clarify the role of the adrenal gland in cobalt polycythemia, a comparison has been made of the effects of intraperitoneal injections of cobalt on the total circulating red cell volume and nucleated erythrocytes of the bone marrow of normal and adrenalectomized dogs.

MATERIALS AND METHODS

Twenty healthy adult female mongrel dogs were used in these studies. They were divided into 4 groups as follows: Group (1) 5 intact untreated, (2) 7 intact dogs given 1.0 mg. elemental cobalt per kg./day i.p., (3) 4 adrenalectomized DCA maintained controls, (4) 4 adrenalectomized DCA maintained dogs receiving 1.0 mg. elemental cobalt per kg./day, two of which were continued for an additional period of time on a combination of 1.0 mg. elemental cobalt and 7 mg. hydrocortisone acetate per kg./day.

Daily rations for all intact dogs consisted of 2 cans of dog food and occasional supplementation with Purina chow. Each dog received an additional supplement of one pound of ground beef twice a week. All dogs were conditioned on an animal farm at least 3 weeks prior to use in an experiment. They were subjected to deworming, distemper vaccination and treatment for mange at least 2 weeks before use. Blood was drawn from the unanesthetized dog without stasis from either the jugular or radial vein and delivered to a heparinized tube. Erythrocyte counts were done using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Wintrobe hematocrit tubes which were spun for 30 minutes at 3000 r.p.m. at a radius of 17.5 cm. Hemoglobin determinations were made on the Klett-Summerson Photoelectric Colorimeter using the acid hematin method (3). Each dog was weighed at the time of the determination of the various blood values.

The red cell volume was determined with P^{32} tagged erythrocytes by the method of Hevesy *et al.* (4) as modified by Krieger *et al.* (5). Ten ml. of a tagged red cell suspension was injected via jugular or radial vein of the unanesthetized dog. After allowing 10 minutes for the sample to mix, 4–5 ml. of blood was removed from the opposite vein for wet counting on an end window Geiger-Mueller counter. Cobalt chloride ($CoCl_2 \cdot 6H_2O$)[2] was given daily as an intraperitoneal injection at a dosage of 1.0 mg. elemental cobalt (4 mg. $CoCl_2 \cdot 6H_2O$) per kg. of body weight.

Bilateral adrenalectomy was performed on the fasted dog under Nembutal[3] anesthesia using aseptic technique. As soon as the first adrenal was removed, 5 mg. desoxycorticosterone acetate, 50 mg. cortisone acetate, and 600,000 units of aqueous procaine penicillin suspension were injected intramuscularly. Immediately following the removal of the second gland both incisions were rinsed with Zephiran[4] and 125 ml. of warm, sterile saline-glucose solution was injected into the jugular vein. The solution contained 0.9% NaCl plus 5% glucose. For 3 days following the operation 5 mg. desoxycorticosterone and 25 mg. cortisone per day were given intramuscularly. The cortisone was then discontinued and the dosage of desoxycorticosterone reduced by 1 mg. per day, until the dog was receiving 1 mg. per day. The adrenalectomized dogs were maintained on 1 mg. desoxycorticosterone per day i.m. plus 2 grams NaCl added to the ration each day. If at any time during the experiment the dogs refused food on two consecutive days, 50 mg. cortisone and 5 mg. desoxycorticosterone were administered i.m. and the dosage of DCA gradually reduced over the next few days. Body ulcers developing in the long term adrenalectomized dogs were successfully combated with sulfadiazine ointment. At

[2] Analytical grade $CoCl_2 \cdot 6H_2O$ with maximum limits of impurity for lead of 0.005%.

[3] 0.5 ml./kg. Abbott Veterinary Nembutal —60 mg./ml.

[4] Winthrop-Stearn Zephiran Chloride solution diluted 1:1,000.

the end of the study the adrenalectomized dogs were autopsied and examined for evidence of accessory adrenals. P values were determined by using Fisher's "t" test for statistical significance.

Bone Marrow Technique.—The dogs were killed with ether and immediately after death the lower three fourths of the femur was dissected and sawed free on both sides. The femurs were placed in a vise and a one quarter inch cross section 25 mm. from the terminal end sawed off and placed in zenker-formol fixative to be prepared for differential cell count. The remaining part of the femur was used for the total nucleated cell count. The total nucleated cell count was done on the fresh marrow squeezed out of the terminal end of each femur. Two successive 120 mg. samples of bone marrow from each femur were weighed on a watch glass and quantitatively transferred to a ground glass homogenizer and diluted with 3 ml. of 2% acetic acid solution. After homogenizing the bone marrow suspension, a sample was transferred to a counting chamber with a red cell pipette and a count made from the 4 corner squares.

Calculations.—The average of the 4 bone marrow samples was inserted into the following formula:

$$\text{No. Nucleated Cells per mg.} = \frac{\text{Average No. Nucleated Cells in 4 squares}}{4} \times 10 \times \frac{3000}{120}.$$

The 3 ml. dilution factor was found to be an acceptable dilution for hypoplastic, hyperplastic and bone marrow of normal cellularity. The bone marrow section for differential cell counting after fixation in zenker-formol solution was washed, decalcified in formalin and trichloroacetic acid, dehydrated in alcohol, cleared in chloroform, embedded in paraffin, sectioned at 5μ, mounted and stained with giemsa. Five hundred cells were differentiated from the finished preparation with a lined square grid in the eye piece under oil immersion lens.

RESULTS

Peripheral Blood.—Changes in body weight, hematocrit, red cell count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and total circulating red cell volume after cobalt injections in normal and adrenalectomized dogs are shown in table 1.

Cobalt injections in the nonadrenalectomized dog (Table 1) induced a significant elevation in hematocrit, red cell count and hemoglobin. After 30 days (15 days in one animal) of treatment the hematocrit had increased from 42.4 ± 3.6 to $52.1 \pm 2.5\%$, ($P < .01$); the erythrocyte count, from $6.7 \pm .87$ to 9.2 ± 1.5 million cells per cu. mm.($P < .01$); the hemoglobin, from 15.5 ± 1.4 to 17.3 ± 1.3 gm. per 100 ml. ($P = .03$). The MCV was decreased from 64.5 ± 5.1 to 58.0 ± 7.9 cubic microns; however, this decrease in cell size was not significant ($P = .1$). The MCH and MCHC were decreased in most instances under cobalt treatment. The red cell volumes expressed as ml. RBC per kg. original body weight (Fig. 1) for the group of intact dogs receiving cobalt for 30 days had a mean value of 46.1 ± 6.6 ml. per kg., which was significantly ($P < .01$) higher than the mean of 36.0 ± 5.6 for nonadrenalectomized untreated dogs.

Cobalt injections failed to cause an elevation in blood values (Table 1) in adrenalectomized dogs as compared with cobalt treated nonadrenalec-

TABLE 1. THE BLOOD PICTURE OF INTACT AND ADRENALECTOMIZED FEMALE DOGS TREATED WITH COBALT OR A COMBINATION OF COBALT AND HYDROCORTISONE

Treatment	No. dogs	Day exp.	Body wt. (kg.)	Hemat.	RBC	HB	MCV	MCH	MCHC	Total RBC volume	Red cell volume (ml./kg.)
Nonadrenalectomized											
I. Untreated	5	0	12.0± .77	43.6±4.4	6.5± .90	15.2±1.4	67.1± 3.0	23.6±2.8	35.0±2.5		
	5	10	11.6± .94	41.6±2.6	6.4± .75	14.6± .86	65.7± 8.5	23.1±4.0	35.5±1.9		
	5	20	12.1±1.2	40.2±5.4	6.5±1.4	14.3±1.6	61.8± 8.8	22.0±3.0	35.6±1.4		
	5	30	12.5±2.1	40.6±4.0	6.0±1.2	14.3±2.2	68.7± 7.5	24.1±3.1	35.2±2.3	451.6±112.2	36.0±5.6
II. Cobalt*	7[1]	0	12.7±2.0	42.4±3.6	6.7± .87	15.5±1.4	64.5± 5.1	23.4±2.7	36.2±2.2	447.7±114.0‡	38.9±4.4
	7	10	12.7±1.9	48.7±2.2	8.6±1.1	16.8±1.0	58.1±10.4	20.0±3.7	34.5±1.8		
	6	20	12.2±1.5	51.7±3.2	9.8± .88	17.8±1.4	53.2± 6.4	18.4±2.3	34.5±1.2		
	6	30	12.5±2.2	52.1±2.5	9.2±1.5	17.3±1.3	58.8± 7.9	18.9±2.5	32.8±2.2	590.0±131.0	46.1±6.6
Adrenalectomized DCA 1 mg./kg./day											
III. Untreated	4	0	13.4±2.4	42.8±3.3	7.6±1.6	14.7±2.0	58.5±15.2	19.9±3.9	34.4±2.3		
	4	10	12.4±1.6	38.8±6.5	6.6± .88	13.7±1.9	59.1±12.0	20.9±3.1	35.4± .97		
	3	20	11.7± .91	42.3±4.1	6.6± .90	13.6±1.9	64.8±16.6	20.7±2.4	32.1±2.9		
	2	30	11.0± .50	42.0±3.2	6.5± .64	13.7±1.6	65.0± 2.0	20.6± .30	32.5± .70		
	2	40	10.7± .70	35.5±4.9	5.7± .78	12.5±1.1	63.0± 0.0	22.2±1.2	35.5±1.8		
	1	50	12.5	28.0	6.0	10.4	46.5	17.4	37.0	348.7± 34.6§	29.7±2.6
IV. Cobalt*	2	0	11.6± .60	40.5±3.5	5.9± .60	12.9±2.4	68.5± 1.5	21.7±1.9	31.6±2.2		
	2	10	12.1± .30	31.0±1.0	4.0±1.3	9.9± .20	85.8±24.2	27.6±8.2	31.8± .60		
	2[1]	20	12.8±1.1	32.0±0.0	6.7± .90	11.3±1.2	48.5± 6.5	17.4±4.2	35.1±3.0	369.7± 24.1	29.3±4.3
	2	30	11.6± .60	34.0±3.0	5.2± .20	12.5±1.4	65.3± 3.2	23.9±1.9	36.7± .90		
	2	40	11.6± .10	30.0±4.0	4.3± .20	11.1±1.4	71.0±12.0	26.2±4.2	37.1± .30		
	2	50	11.7± .20	29.5±2.5	4.3± .40	9.8± .90	68.8± .80	22.8±0.0	33.2± .20	318.9± 21.2	27.4±2.4
V. Cobalt* Hydrocortisone†	2	0	14.1± .30	43.0±3.0	8.0± .40	15.3±1.1	54.0± 6.5	19.3±2.3	35.5± .10		
	2	10	13.8±2.0	33.0±3.0	5.6±1.3	11.6±2.0	61.0± 9.0	20.8±1.2	34.7±3.1		
	2	20	13.6±1.2	36.0±4.0	6.0± .90	14.3±1.1	61.0± 2.0	24.1±1.7	39.7±1.5		
	2	30	12.1±2.1	43.0±7.0	6.2±1.0	15.9±2.6	69.3± .20	25.6± .20	36.9± .10		
	2[1]	40	11.2±2.1	39.0±7.0	6.0± .40	14.3±3.1	64.0± 8.0	23.5±3.7	36.4±1.4	328.1± 38.5	29.9±2.3
	2	50	11.1±1.1	38.5±7.5	6.0± .40	13.9±1.7	65.0± 7.0	22.8±1.6	36.5± .50		
	2	60	9.8±1.4	37.0±1.0	5.5± .10	13.2± .50	66.8± 3.7	23.6± .10	35.6±1.8		
	2[2]	70	8.9± .70	31.0±2.0	5.1± .70	10.0±0.0	62.0± 4.0	20.2±2.6	32.5±2.0	318.9± 32.5	34.4±2.2
	2	80	9.4±1.7	30.0±9.0	4.9±1.7	9.4±3.1	62.8± 2.7	19.5± .20	31.0±1.0		
	1	90	9.5	32.0	4.1	11.4	78.0	27.8	35.6		
	1	100	10.6	30.0	5.1	11.2	59.0	22.0	37.4		
	1	110	10.1	34.0	6.6	12.5	51.5	19.0	36.8		
	1	117	11.6	34.0	5.6	12.0	60.5	21.4	35.2	359.1	35.6

DAY = Day of experiment, BW = Body weight in kg., HEMAT = Hematocrit in per cent, RBC = Red Cell Count in millions per cu. mm., HB = Hemoglobin in g. per 100 ml., MCV = Mean corpuscular volume in cu microns, MCH = Mean corpuscular hemoglobin in micromicrograms, MCHC = Mean Corpuscular Hemoglobin Concentration in per cent, Total Red Cell Volume expressed in ml., ± = Standard Deviation.

* 1.0 mg. elemental cobalt/kg./day i.p.

† 7.0 mg. hydrocortisone/kg./day i.m.

‡ Mean initial red cell volumes for 3 of the 7 dogs.

§ = Mean red cell volumes for 3 dogs 20, 40 and 50 days respectively after adrenalectomy.

[1] = Cobalt chloride injections started i.p.

[2] = Cobalt chloride injections continued i.p. in addition to hydrocortisone i.m.

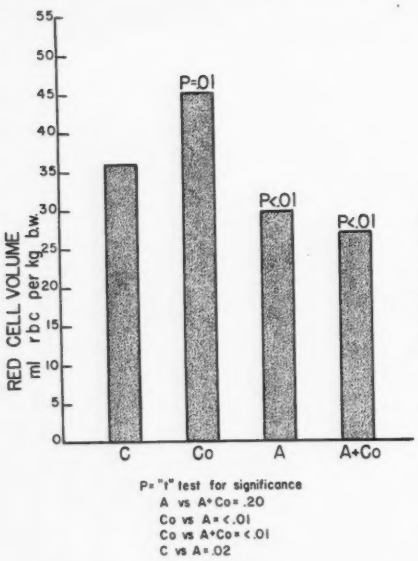

FIG. 1. Mean values for total circulating red cell volume expressed as ml. RBC per kg. body weight in intact untreated dogs (c), intact dogs treated with cobalt for 30 days (Co), DCA maintained adrenalectomized dogs (A) and DCA maintained adrenalectomized dogs treated for 30 days with cobalt (A+Co). P values over each bar indicate the significance in comparison with intact untreated dogs (c).

tomized dogs. In fact the blood values of cobalt-treated adrenalectomized dogs showed a progressive decrease as in adrenalectomized DCA maintained dogs. After 30 days of cobalt treatment in 4 adrenalectomized dogs (Table 1, Groups IV and V), the hematocrit had decreased from 35.5 ± 7.0 to $30.0 \pm 2.8\%$; the erythrocyte count from $6.4 \pm .88$ to $4.7 \pm .77$ million cells per cu. mm.; the hemoglobin, from 12.8 ± 3.2 to $10.0 \pm .77$ gm. per 100 ml. The mean MCV value for the 4 adrenalectomized dogs treated for 30 days with cobalt was 65.4 ± 5.2 as compared with 67.1 ± 3.0 for untreated nonadrenalectomized dogs and 64.8 ± 16.6 for DCA maintained adrenalectomized dogs 20 days after adrenalectomy. MCH values in adrenalectomized dogs treated with cobalt for 30 days were decreased in most instances while the MCHC was not significantly different from untreated nonadrenalectomized or DCA maintained adrenalectomized dogs. The red cell volume in 4 adrenalectomized dogs receiving cobalt for 30 days (Fig. 1) was 27.0 ± 3.3 ml. per kg. and significantly less ($P < .01$) than the mean of 36.0 ± 5.6 for 5 intact, untreated dogs but not significantly different ($P = .2$) from the value of 29.6 ± 3.2 for 7 DCA-maintained adrenalectomized dogs prior to treatment. A slight loss in body weight was noted in adrenalectomized dogs being treated with cobalt.

Two of the 4 adrenalectomized dogs (Table 1, Group V) treated for 30 days with cobalt were continued for an additional period on a combination of 1.0 mg. elemental cobalt as $CoCl_2 \cdot 6H_2O$ and 7 mg. hydrocortisone acetate per kg./day. One dog treated for an additional 57 days with the combination showed a moderate elevation in hematocrit (17%), erythrocyte

TABLE 2. DIFFERENTIAL BONE MARROW CELL COUNTS (MYELOGRAMS) IN INTACT AND ADRENALECTOMIZED DOGS TREATED WITH COBALT OR A COMBINATION OF COBALT AND HYDROCORTISONE (VALUES EXPRESSED AS PER CENT OF TOTAL CELLS ENUMERATED)

Treatment	No. dogs	Erythroid			Myeloid			Stem	Erythroid: myeloid ratio
		Early	Late	Total	Eosinophiles	Neutrophiles	Total		
Intact									
Untreated	8	7.8±1.9	33.7±3.4	41.5±3.0	2.4±1.4	55.0±3.4	57.3±3.2	1.2±.90	0.73±.09
Cobalt*	3	8.7±3.9	55.8±8.5	64.5±6.2	2.2±1.0	32.1±2.8	33.0±5.6	1.3±.90	1.94±.51
Adrenalectomized—DCA 1 mg./kg./day									
Untreated†	3	2.9±1.4	13.3± .65	16.2± 1.6	4.7±3.2	77.4± 5.6	82.1± 2.7	1.7±1.8	.20±.025
Cobalt*	2	1.2± .10	12.3± 2.9	13.5± 3.1	.8± .50	84.7± 1.8	85.6± 2.3	.8± .87	.16±.042
Cobalt‡ Hydrocortisone	2	8.4±3.2	25.8±10.3	34.1±13.4	0	62.8±14.7	62.8±14.7	3.2±1.8	.59±.035

* Cobalt Chloride at a dosage of 1.0 mg. elemental cobalt per kg./day for 30 days (i.p.)
† DCA maintained for 20, 40 and 50 days respectively. ± = Standard Deviation.
‡ Cobalt Chloride (1.0 mg. elemental cobalt/kg./day for 30 days i.p.) and a combination of cobalt chloride and hydrocortisone (7 mg./kg./day i.m.) for 57 days in one dog and for 20 days in a second dog.

count (27%), hemoglobin (20%) and total circulating red cell volume (2%). A second dog treated for an additional 20 days with the combination, with blood values being determined only after 10 days, also showed an elevation in hematocrit (18%), erythrocyte count (14%) and hemoglobin (25%).

An additional 5 intact dogs were injected with 2.5 mg. elemental cobalt per kg./day i.p. and none survived longer than 12 days. Two intact dogs were injected with 1.0 mg. elemental cobalt per kg./day subcutaneously and after 8–10 days necrosis and sloughing of the skin were noted at the injection site. The lack of toxicity of the lower dosages of cobalt when given via an intraperitoneal injection is apparently the result of its being spread out over a larger surface area, resulting in a more rapid absorption from the injection site. Three adrenalectomized dogs were injected i.p. with a dosage of 2.5 mg. elemental cobalt per kg./day and none survived longer than 5 days. The adrenalectomized animal is known to be more susceptible to toxic agents than the intact animal (6). No adrenalectomized dog was found with accessory adrenals at autopsy.

A method is also given for quantitating total nucleated cellularity of bone marrow, which varies from other techniques previously reported for quantitatively assessing total cellularity of bone marrow. The small variation in counts among intact untreated dogs (Table 3) indicates that within limits this technique is a reliable method for estimating quantitatively total nucleated cells of bone marrow. A further estimate of the total myeloid, erythroid and stem cells of bone marrow can be made by determining the percentage of the various types of bone marrow cells from the corresponding bone marrow section. Such a technique seems useful in that myelogram percentages alone may accentuate unimportant relationships and yield no information concerning the actual numbers of cells present. Volumetric techniques are available for quantitating bone marrow cellularity but have the disadvantage of diluting the bone marrow tissue excessively when a fatty bone marrow is encountered.

Bone Marrow Cytology.—In order to understand the significant changes which occur in the periperal blood of the dog during the administration of cobalt or a combination of cobalt and hydrocortisone, the status of the bone marrow seemed of utmost importance. The results of a study of the myelograms and total nucleated cellularity of bone marrows taken from some of the dogs seen in Table 1 are shown in Tables 2 and 3. The total ertythroid percentages and erythroid: myeloid ratios for nonadrenalectomized dogs receiving cobalt (Table 2) were significantly greater ($P < .01$) and total myeloid percentages were significantly less ($P < .01$) than in nonadrenalectomized untreated dogs. As seen in Table 3; the significant increase ($P < .01$) in total nucleated cells from the bone marrow of nonadrenalectomized cobalt-treated dogs was due entirely to an increase in total erythroid cells with no apparent change in total myeloid cells. The

TABLE 3. THE EFFECTS OF COBALT AND A COMBINATION OF COBALT AND HYDROCORTISONE ON THE TOTAL NUCLEATED CELLS OF THE BONE MARROW OF NORMAL AND ADRENALECTOMIZED DOGS

Treatment	No. dogs	Total nucleated cells	Total erythroid cells	Total myeloid cells	Total stem cells
		Thousands per mg. bone marrow tissue			
Intact					
Untreated	6	34.2± 3.8	14.1±1.9	19.6±2.3	.5±.6
Cobalt*	5	59.4±18.2	38.6±8.1	19.4±4.7	1.2±.88
Adrenalectomized—DCA/mg./kg./day					
Untreated	3	43.8± 9.8	7.1±1.9	36.1± 8.6	.51±.49
Cobalt*	2	39.6±22.8	5.0±1.9	34.2±20.4	.90±.32
Cobalt†+Hydrocortisone	2	24.6± 8.4	7.5± .97	16.0± 8.7	1.19±.60

* Cobalt Chloride at a dosage of 1.0 mg. elemental cobalt per kg./day for 30 days (i.p.).
† Cobalt Chloride (1.0 mg. elemental cobalt/kg./day for 30 days i.p) and a combination of cobalt chloride and hydrocortisone (7 mg./kg./day i.m.) for 57 days in one dog and for 20 days in a second dog.
± =Standard Deviation.

increase in total erythroid cells was almost exclusively due to the late erythroid series or the orthochromatic normoblast as seen in Figure 3. The total stem cells were also increased in nonadrenalectomized dogs treated with cobalt.

DCA-maintained adrenalectomized dogs had bone marrows with a significant ($P < .01$) increase in total myeloid cells (Table 3) which was due largely to an increase in neutrophilic metamyelocytes, bands and segmenters. The observations on the bone marrow sections reflect changes observed in the myelograms (Table 2). Thus, a decrease in the erythroid elements and an increase in the relative numbers of myeloid cells shown in tables 2 and 3 are even apparent from a gross inspection of Figure 4. A significant decrease in the erythroid: myeloid ratio ($P < .01$) occurred in bone marrows from DCA-maintained adrenalectomized dogs. No significant change occurred in the relative numbers of stem cells in bone marrows from long term DCA-maintained adrenalectomized dogs.

Two DCA-maintained adrenalectomized dogs treated for 30 days with cobalt had bone marrow myelograms and total number of nucleated cells which were not significantly different from those of DCA-maintained adrenalectomized dogs. These findings confirm the total circulating red cell volume data showing that cobalt does not stimulate erythropoiesis in the adrenalectomized dog. The total number of erythroid cells and erythroid: myeloid ratios (Tables 2 and 3) appeared to be lower in bone marrows from adrenalectomized dogs treated with cobalt (Fig. 5) than those of DCA-maintained adrenalectomized dogs but the differences were not significant.

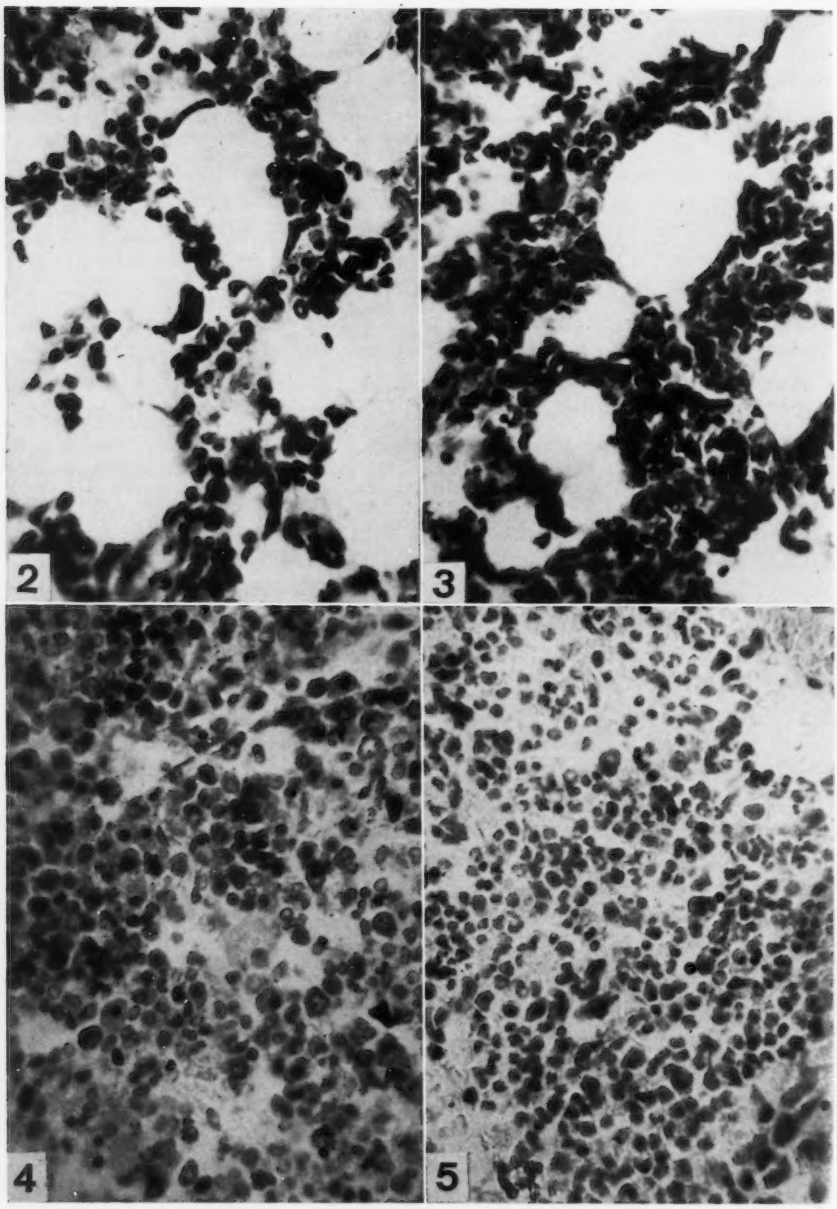

FIG. 2. Femur marrow from a nonadrenalectomized untreated dog ×143. Not moderate amount of fat cells and a few dark islands of cells representing erythroi elements.

FIG. 3. Femur marrow from a nonadrenalectomized dog treated daily for 30 days wit

Two adrenalectomized dogs that had been treated for 30 days with cobalt and had shown no significant change in red cell volume were continued for an additional period (57 days in one and 20 days in a second dog) on a combination of cobalt and hydrocortisone. As seen in Table 1 an elevation in hematocrit, hemoglobin and red cell count resulted along with a slight to moderate increase in total circulating red cell volume. Both dogs showed some change (Tables 2 and 3) in the total number of myeloid cells after treatment with the combination. The results in Table 2 show an increase approaching a return to normal of the erythroid: myeloid ratio in the bone marrows taken from adrenalectomized dogs treated with both cobalt and hydrocortisone concurrently. Another point which desreves attention is the reappearance of the vacuolated fat cells (Fig. 6) in the bone marrow of an adrenalectomized cobalt-treated dog injected for an additional period with a combination of cobalt and hydrocortisone.

DISCUSSION

Some investigators of cobalt polycythemia have concluded that the normal dog, when given cobalt orally, fails to respond with a significant elevation in blood values (7, 8) similar to other species (9, 10, 11, 12). Brewer (13) statistically analyzed the results of several cobalt feeding experiments and found that a significant increase in red cell count, hemoglobin and hematocrit did occur when the daily oral dose exceeded 15 mg. per kg. Evidence is presented which establishes proof that the intact dog will respond to intraperitoneal injections of cobalt with a marked elevation in total circulating red cell volume. The increase in total erythroid cells and erythroid:myeloid ratios of bone marrow from intact dogs treated with cobalt confirm the elevation in total circulating red cell volume and are further proof that cobalt is capable of stimulating hematopoiesis in normal dogs.

It has been found that the adrenalectomized rat shows a moderate anemia for the first 20 days after adrenalectomy (14, 15, 16) which is corrected upon the development of accessory adrenals (16). The rat recovers from the anemia of adrenalectomy after 30 days (16), whereas the DCA maintained adrenalectomized dog still remains anemic 50 days after adren-

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1.0 mg. elemental cobalt per kg. ×143. Note increased cellularity along with the greater concentration of dark staining erythroid islands. Also note smaller number of vacuolated fat cells in comparison with the normal control in Figure 2.

FIG. 4. Femur marrow from adrenalectomized—DCA (1.0 mg./kg./day) maintained dog 22 days after adrenalectomy ×143. Note the markedly increased myeloid cellularity and complete disappearance of erythroid islands.

FIG. 5. Femur marrow from an adrenalectomized dog maintained for 20 days on 1.0 mg. DCA/kg./day and treated for the next 30 days with 1.0 mg. DCA and 1.0 mg. elemental cobalt/kg./day ×200. Note increased myeloid cellularity and lack of definitive erythroid islands.

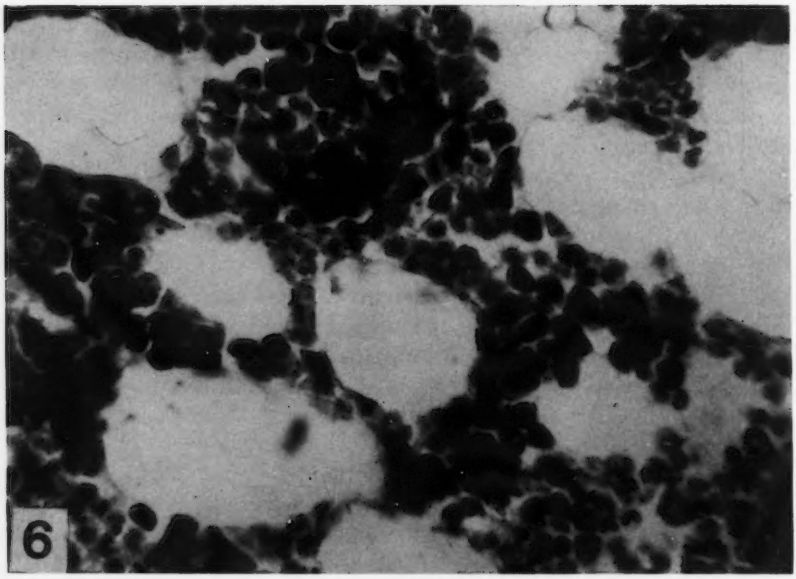

FIG. 6. Femur marrow from an adrenalectomized dog maintained for 40 days on 1.0/mg. DCA/day, treated for the next 30 days with 1.0 mg. DCA and 1.0 mg. elemental cobalt per kg./day and the next successive 57 days with DCA, cobalt and 7 mg. hydrocortisone per kg./day×143. Note return of the normal appearance of erythroid islands and vacuolated cells similar to the normal dog in Figure 2.

alectomy and will go into severe adrenal insufficiency and die within a few days if the DCA injections and dietary salt supplementation are withdrawn. The failure of the adrenalectomized dog to develop accessory adrenals may explain the inability of the dog to correct the anemia of adrenalectomy. The red cell volume in adrenalectomized dogs with or without cobalt is significantly less than those of intact untreated dogs. The difference in red cell volumes in DCA maintained adrenalectomized dogs and adrenalectomized dogs treated for 30 days with cobalt is not significant, thus indicating that the removal of the adrenals in dogs renders them incapable of responding to cobalt injections with an elevation in blood values.

Swingle *et al.* (24) have reported that the low values for hemoglobin, hematocrit and erythrocyte count which characterize adrenalectomized dogs, in some instances, are increased to normal by long continued massive doses of cortisone. Administration of hydrocortisone or corticosterone to normal rats over a prolonged period of time induced a significant elevation in total circulating red cell volume (17) and the elevation in red cell volume achieved in adrenalectomized rats treated with concurrent injec

tions of cobalt and hydrocortisone was significantly greater than the additive effects of either substance injected alone (18). Hydrocortisone and cobalt, when injected concurrently into dogs, improved the hematocrit, hemoglobin, erythrocyte count and total red cell volume to some degree. It seems quite likely that a favorable combination of cobalt and hydrocortisone can be found in which the "synergistic" action of the two drugs can be demonstrated to the same degree as with adrenalectomized rats.

The failure of adrenalectomized dogs to respond to cobalt injections with a change in red cell volume is interpreted to mean that the adrenocortical hormones are necessary for the production of the characteristic cobalt polycythemia. The fact that hydrocortisone and corticosterone are capable of stimulating erythropoiesis in the intact rat (17) and that a joint hematopoietic action of a combination of cobalt and hydrocortisone is exerted (18) seems proof that the action of cobalt and the adrenocortical secretions in stimulating erythropoiesis in synergistic.

The decrease in the erythroid: myeloid ratio and increase in total myeloid cells in bone marrow from DCA-maintained adrenalectomized dogs are similar to the findings of Gordon *et al.* (19) for the adrenalectomized rat. Fruhman *et al.* (20) found that the administration of corticosterone to adrenalectomized rats resulted in a significant increase in the number of nucleated erythroid cells and a decrease in the number of neutrophiles and eosinophiles within femoral bone marrows. The total erythroid and myeloid percentages for the intact untreated dog shown in Table 2 are very similar to bone marrow myelograms from 187 normal adult dogs reported by Albritton (21). Cobalt injections in adrenalectomized dogs induced no significant change in bone marrow myelograms. However, injections of a combination of cobalt and hydrocortisone into adrenalectomized dogs partially restored to normal the erythroid:myeloid ratio and the total nucleated cellularity of the bone marrow.

The evidence seems to suggest that cobalt does not produce polycythemia by a direct effect on bone marrow itself (22) but acts secondarily through an elevation in plasma erythropoietin (23). The most reasonable hypothesis at this time, to explain the role of the adrenal secretions in cobalt polycythemia, is that the adrenocortical hormones act synergistically with erythropoietin to stimulate the blood forming organs.

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CORTICOSTEROIDS AND THE FUNCTIONAL CAPACITY OF THE RAT HEART-LUNG PREPARATION[1,2]

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ABSTRACT

A technic has been developed for the preparation and maintenance of the isolated rat heart-lung. Index of left ventricular work expressed as cardiac output times mean arterial blood pressure was highly reproducible for a given set of experimental conditions. Order of work performance arranged from highest to lowest was as follows: 1) heart-lung from intact rat perfused with blood from intact rats, 2) heart-lung from adrenalectomized rat perfused with blood from intact rats, 3) intact-adrenalectomized, 4) adrenalectomized-adrenalectomized. Corticosterone, but not Substance S, added to the perfusion blood of the intact-adrenalectomized preparation restored work capacity toward that of the intact-intact preparation.

FUNCTIONAL impairment of the cardiovascular system is a prominent feature of adrenal insufficiency (1, 2, 3, 4), the nature of which has been the subject of numerous investigations. *In vivo* studies suggest that the impairment involves reduction in the contractile force of the myocardium (5) as well as inadequacy of peripheral vascular functions (6). *In vitro* studies designed to more clearly define the influence of the corticosteroids on the heart have included the isolated perfused heart and the muscle strip. The diversity of results and the relatively high concentrations of steroids employed make it impossible to reach definitive conclusions as to the action of the secretions of the adrenal cortex on the myocardium (7). For example, Nasmyth (7) found a depressant action of the corticosteroids on guinea pig and rat hearts perfused by the Langendorf technic, whereas Tanz *et al.* (8) described a positive inotropic action of 9α-fluorohydrocortisone on the isolated cat papillary muscle and on the isolated perfused rat heart.

The present study had two objectives: 1) to establish conditions for high reproducibility of work performance of the isolated rat heart-lung and 2)

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to examine the possibility that the corticosteroids have an influence on the functional state of this isolated preparation. Work performance has been selected as the index of functional state because of the relative ease with which it may be measured (pressure times cardiac output).

ANIMALS

Male rats, 300 to 350 gm. in body weight, were obtained from the Holtzman Farm. Bilateral adrenalectomy was performed two to three weeks before the isolation of the heart-lung; control animals were subjected to sham adrenalectomy. The anesthetic for all surgical procedures was sodium pentobarbital, 40 mg./kg., administered intraperitoneally as a one per cent aqueous solution. Adrenalectomized animals were maintained post-operatively on 0.9% sodium chloride solution; controls drank tap water. Both groups were housed in a constant temperature room for at least two weeks before isolation of the heart-lung. They were fed Friskies dog chow *ad libitum*. Completeness of adrenalectomy was checked by inspection of the suprarenal area.

HEART-LUNG PREPARATION

The isolated rat heart-lung preparation was similar to that described by Knowlton and Starling for the dog (9). In the preparation to be described the lungs were collapsed and not functional but the pulmonary vessels were part of the circuit. Blood was conducted by gravity in a thin film over a stainless steel screen in an atmosphere of 95% oxygen, 5% carbon dioxide and collected at the bottom of the oxygenator[5] which served as the "venous reservoir." In a series of preliminary observations it was demonstrated that positive pressure respiration induced pulmonary edema which was a major factor in limiting the survival of the preparation. Extrapulmonary oxygenation significantly prolonged survival time and maintained concentrations of arterial and venous oxygen of approximately 15 and 12 volumes per cent, respectively.

The course of the blood in the system was in brief: right heart, pulmonary vascular bed, left heart, aorta, innominate artery, bubble flowmeter, screen oxygenator, coiled tube immersed in constant temperature bath (37° C), inferior vena cava and back to right heart.

A capillary mercury manometer was connected by a limb of a T-tube to the circuit just beyond the carotid artery catheter. A tunnel clamp was employed to adjust peripheral resistance and was located in the circuit immediately before the bubble flowmeter.[6]

The operational sequence for the conduct of an experiment was as follows. 1) The venous reservoir was charged with 50 ml. of fresh heparinized blood obtained from anesthetized donor animals. 2) The trachea of an anesthetized experimental animal was connected to a positive pressure respirator. 3) A midline incision was carried from the symphysis pubis through the manubrium. 4) The thoracic walls were retracted. 5) The right and left superior vena cavae, the left common carotid and both subclavian arteries securely ligated. 6) Two loose ligatures were placed under the right common carotid artery high in the neck. 7) One loose ligature was placed about the thoracic vena cava and another about the arch of the aorta just distal to the origin of the innominate artery. 8) Heparin sodium (1.5 mg.) was injected into the left renal vein. 9) The distal ligature under the right common carotid artery was tied. 10) A small artery clamp was placed on the innominate artery. 11) A catheter was inserted into the right carotid artery.

[5] Dr. Richard Eckstein kindly loaned us this modified Gibbon oxygenator which consits of a 2×12×6″ rectangular lucite container in which is mounted a stainless steel wire screen.

[6] The authors will supply details about the plastic tube connections upon request.

advanced into the innominate artery and secured with a ligature. 12) Initial infusion pressure (venous pressure) was fixed at 10 cm. of water by adjustment of the level of blood in the reservoir. In rapid succession 13) the inferior vena cava was cannulated at the point where the cava passes through the diaphragm, 14) the aortic arch ligated, 15) the inferior vena cava catheter secured with a ligature, 16) stopcocks, one on the inflow, the other on the ouflow tubing, were opened, 17) artificial respiration was terminated.

The procedures must be performed in a relatively uniform manner, in less than 10 minutes, and with minimal hemorrhage. After the stopcocks were opened, an initial period of instability, less than one minute in duration, characterized by cardiac arrhythmia and unstable arterial pressure, was succeeded by a stable period of variable length. During the stable period, mean arterial pressure was maintained at 80 mm. Hg in Exp. 1 and 110 mm. Hg in Exp. 2 by adjustment of the peripheral resistance clamp. During the stable period, cardiac output, mean arterial blood pressure, and temperature of venous blood were periodically recorded or adjusted.

Left ventricular work index was calculated as follows:

$$W = K\frac{(\text{C.O.})\ (P)\ (T)}{M},$$

where W = the left ventricular work index in gram-meters per 100 mg. dry heart weight, K = a constant composed of conversion factors, C.O. = cardiac output in ml. per min., P = pressure in mm. Hg, T = time in minutes that the preparation was able to maintain a predetermined level of blood pressure, and M = dry heart weight expressed in mg.[7]

RESULTS

In Table 1 are presented the results of Experiment 1 in which peripheral resistance was adjusted to maintain a mean arterial pressure of 80 mm. Hg. Three preparations were studied: 1) heart-lung from an intact rat perfused with blood from intact rats (intact-intact), 2) heart-lung from an adrenalectomized rat perfused with blood from adrenalectomized rats (adrenex-adrenex), and 3) heart-lung from an intact rat perfused with blood from adrenalectomized rats (intact-adrenex). The work index (work of the left ventricle during the period in which the preparation was capable of maintaining a pressure of 80 mm. Hg) was influenced by the secretions of the adrenal gland. The work index of the heart-lung from either adrenalectomized rats or intact rats was at the same low level when the perfusion blood was obtained from adrenalectomized rats. The results suggest that impairment of functional activity was a consequence of low titers of corticosteroids and/or epinephrine or of toxic factors in blood from adrenalectomized rats.

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[7] The calculated left ventricular work index is not the total work performed by the left ventricle. Not included is 1) the work performed in accelerating the blood in its passage from the ventricle into the aorta and 2) that part of the cardiac output which is directed into the coronary arteries. In the intact heart, coronary flow is usually a relatively small fraction of the total cardiac output. However, in the isolated heart-lung, coronary flow may represent a relatively high fraction of total cardiac output. We have no experimental data to evaluate changes in coronary flow in this study. For that reason we have described our calculation of cardiac performance as an "index."

TABLE 1. LEFT VENTRICULAR WORK INDEX OF RAT HEART-LUNG (EXP. 1)[1]

Type of preparation	Intact-Intact[3]	Adrenex-Adrenex[4]	Intact-Adrenex[5]
Number of experiments	12	12	6
Work Index[2] ±S.E.	444 ±56	126 ±31	136 ±45

[1] Mean arterial blood pressure = 80 mm. Hg. No glucose added.
[2] Expressed as gram-meters per 100 mg. dry heart weight.
[3] Intact-Intact = heart-lung preparation from intact rat perfused with blood from intact rats.
[4] Adrenex-Adrenex = heart-lung preparation from adrenalectomized rat perfused with blood from adrenalectomized rats.
[5] Intact-Adrenex = heart-lung preparation from intact rat perfused with blood from adrenalectomized rats.

The total work index does not reveal certain characteristics of the three preparations. For that reason, cardiac output against time has been plotted in Figure 1 for three typical experiments. The marked decrease in cardiac output of the heart-lung from the adrenalectomized rat is apparent at the beginning of the experiment. In the case of the intact-adrenex preparation the cardiac output is initially relatively high.

The results of Experiment 2 in which mean arterial pressure was maintained at 110 mm. Hg are presented in Table 2. An additional preparation, adrenex-intact was included. Glucose was added at the beginning of the experiment to the perfusion blood in a concentration of 100 mg. per 100 ml. At the higher level of pressure the work index of the intact-intact preparation was less than that of the same preparation operating at a pressure of

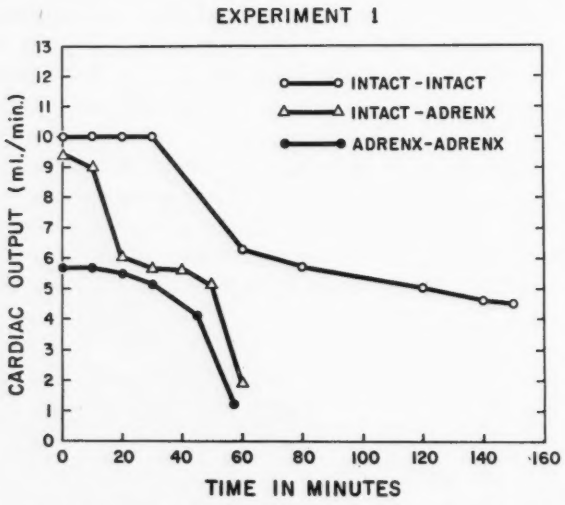

FIG. 1. Cardiac output plotted against time for three typical preparations in experiment 1. Area under each curve is related by a constant to left ventricular work index.

TABLE 2. LEFT VENTRICULAR WORK INDEX OF RAT HEART-LUNG (EXP. 2)[1]

Type of preparation	Intact-Intact[3]	Adrenex-Adrenex[3]	Adrenex-Intact[3]	Intact-Adrenex[3]		
Amount of steroid added per 100 ml. perfused blood	0	0	0	0	10 μg. Substance S	10 μg. Corticosterone
Number of experiments	18	15	15	15	5	5
Work Index[2] ±S.E.	235 ±24	25 ± 4	75 ±12	50 ± 5	49 ± 5	108 ± 6

[1] Mean arterial blood pressure = 110 mm. Hg, 100 mg. glucose added to each 100 ml. of perfusion blood.
[2] See Table 1 for legend.
[3] See Table 1 for legend.

80 mm. Hg. The impairment in function of the adrenex-adrenex preparation was even more marked than in Exp. 1. Perfusion of the heart-lung from adrenalectomized rats with blood from intact rats had a positive effect on work capacity. In contrast to Exp. 1, the intact-adrenex preparation performed a significantly greater amount of work than the adrenex-adrenex preparation.

Substance S (17α,21-dihydroxy-Δ^4-pregnene-3,20-dione) added to the perfusion blood of the intact-adrenex preparation induced no change in work capacity. On the other hand, corticosterone added in a concentration of 10 μg. per 100 ml., significantly improved the work index but did not restore it to normal.

DISCUSSION

Technics have been described for the preparation of the heart-lung in the rat (10, 11, 12, 13). In the study reported here, uniformity of surgical procedures, health of animals, extrapulmonary oxygenation, constancy of blood pressure, and maintenance of a constant temperature of the perfusion blood have led to the development of a heart-lung preparation which is characterized by a high degree of reproducibility of work performance.

The average cardiac output of the intact-intact preparations in Exp. 1 was 9.9 ml. per min., which is to be compared with 64 ml. per min. for cardiac output in normal rats measured by the direct Fick principle (14). This difference between the isolated heart and the heart *in vivo* in the rat is of the same order of magnitude as that observed in the dog. In the intact animal, coronary flow represents a small fraction of the total work of the left ventricle. In the isolated heart, coronary blood flow, which is not included in work index, may represent a significant fraction of the total cardiac output. Future studies should be directed toward measurement of coronary flow in the isolated rat heart in order to assess work capacity more precisely.

The basis of the impairment of function in the heart-lung deprived of

the secretions of the adrenal is unknown. The following experiment suggests that one factor involved is a change in the functional state of the myocardium. Heart-lung preparations were prepared from intact rats and from rats adrenalectomized at various intervals prior to testing. Both types of preparations were perfused with blood from intact rats. The results indicate that a period of at least two weeks must elapse between adrenalectomy and testing before a significant reduction in work capacity can be demonstrated. Of interest is the fact that after two weeks of adrenal insufficiency myocardial atrophy as measured by dry weight (10% less than normal) is also definitely established. The mechanism of this myocardial atrophy which develops slowly after removal or destruction of the adrenals in the rat and in man (3) is unknown.

No data are available to evaluate the length of time after adrenalectomy which is necessary to manifest the changes in the perfusion blood which induce impairment of function of the heart-lung. The fact that addition of corticosterone in a concentration of 10 μg. per 100 ml. partially restores the functional capacity of the intact-adrenex preparation suggests that at least one acute factor is a deficiency of corticosteroids. However, a toxic factor or a deficiency of epinephrine may also contribute to the poor work performance of the intact-adrenex preparation.

Substance S was ineffective, corticosterone active, in the isolated system. Substance S has no effect on carbohydrate metabolism and has at most a slight influence on electrolyte excretion, whereas corticosterone significantly influences both types of metabolism in the whole animals. Additional studies are planned to relate activity of steroids in the whole animal and in the heart-lung.

The fact that certain corticosteroids are quite effective when administered by the oral route has prompted certain investigators (15, 16) to suggest that the liver exerts an "activating" influence on the corticosteroids. The present study indicates that the liver is not essential for the biological action of corticosterone.

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EXPERIMENTAL DIABETES IN THE CATFISH: NORMAL AND ALLOXAN-DIABETIC BLOOD GLUCOSE AND PANCREATIC HISTOLOGY[1]

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ABSTRACT

Alloxan diabetes has been demonstrated in the common lake catfish by correlated histological and blood sugar studies. Anatomical considerations make this animal particularly suitable for this work. The conus arteriosus is conveniently situated for intravascular injection and serial blood sampling. The concentration of endocrine pancreas in a principal islet and accessory islets, all with limited exocrine tissue, facilitates histological preparations. In 237 determinations, using a Folin-Malmros micro method modified for fish blood, the normal blood sugar value was found to be 59 mg.%, with a standard deviation of 19 mg.%. Alloxan was administered through the conus of 50 fish, at 400 mg./kg. In alloxanized fish, blood sugar levels of 120–200 mg.% were typical. This hyperglycemia persisted through the 96 hour period studied and, in a few fish not sacrificed earlier for histological study, for 14 days, the longest period studied. In the intact animal, the principal islet is composed of many, heavily-granulated beta cells, with a similar number of other islet cells and a cortex of exocrine tissue. Within 3 hours of alloxan injection, degranulation of beta cells was apparent. In subsequent sampling periods, up to 96 hours, degranulation increased, accompanied by progressive hydropic degeneration and nuclear disintegration. These changes were found only in beta cells.

THE mechanism of the diabetogenic action of alloxan has been investigated extensively but without conclusive results. The need for better understanding of this phenomenon has been increased by the Loubatiere hypothesis of the importance of endogenous alloxan in the etiology of human diabetes mellitus. It appears that special features of the lower vertebrates may facilitate observations pertinent to this hypothesis. These forms offer very large cells, favorable to histological or cytochemical study, and comparatively slow physiological responses, which may permit identification of functional changes too rapid for observation in mammals. Certain teleost fish provide the additional advantage of the concentration

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of islet tissue in nodules containing little exocrine tissue. They also provide convenient sites for injection and blood sampling, such as the gill arch of the toadfish or the conus arteriosus of the catfish.

Examination of the scanty literature of experimental diabetes in fish offers little encouragement. Reports are often based on very small numbers of animals and contain many conflicting observations. They agree on 2 points, the extreme variability of the normal blood glucose levels of fish and the comparable variability of response to alloxan.

Lazarow and Berman (8) have reported hyperglycemia and beta cell lesions in the toadfish, *Opsanus*, following alloxan injection. The observations of Nace (11), on the same form, have been less consistent. Grosso (3), using the guppy, *Lebistes*, has produced beta cell lesions in animals maintained in alloxan solutions. This careful and detailed histological study includes no blood sugar data and its interpretation is complicated by the observations of Patterson, Lazarow and Levy (15), of the very short effective life of alloxan under physiological conditions. While Lazarow and Berman mention only beta cell lesions, Grosso reports significant changes in liver, kidney and spleen.

In a series of 4 reports, Saviano (16) has described the effects of alloxan on 3 fish, *Scyllium*, *Motella* and *Scorpaena*. In none of these did he observe beta cell lesions comparable to those reported by Grosso and by Lazarow and Berman. In all 3 forms, alpha cell lesions were the most consistent feature. Hyperglycemia was found in some animals, but with no uniformity. In *Scyllium*, lesions of exocrine cells were a common observation. LaGrutta (7) has confirmed the findings of Saviano on *Scorpaena*.

Similar exocrine tissue damage was observed by Pallot and Schatzle (14), in carp. But these investigators found no consistent change in alpha or beta cells. Doerr (2), with carp and tench, found no histological changes. Neither investigator included any blood sugar data.

In summary, published reports of the action of alloxan in fish indicate that hyperglycemia may occur in some species and that lesions may be found in alpha cells, beta cells, exocrine tissue, liver, kidney and spleen. To clarify some of the factors concerned in these marked differences among genera, an integrated study of histological and blood sugar responses to alloxan among several fish has been undertaken. The present report describes the consistent and progressive hyperglycemia and beta cell destruction found in the alloxanized catfish.

MATERIALS AND METHODS

Ninety adult catfish, *Ictalurus nebulosus*, (18) (200–600 gm. $\pm$ 5 gm.) were placed, in groups of 10, in aquaria provided with aerated, circulating, charcoal-filtered tap water at 10°–15° C. From each tank, 5 fish were randomly selected and injected through the conus arteriosus, as shown in Figure 1, with 10% iced, aqueous alloxan, dissolved less than 5 minutes before use. The remaining 5 fish in each aquarium were injected by the same route with similar volumes of distilled water and served as controls for blood and

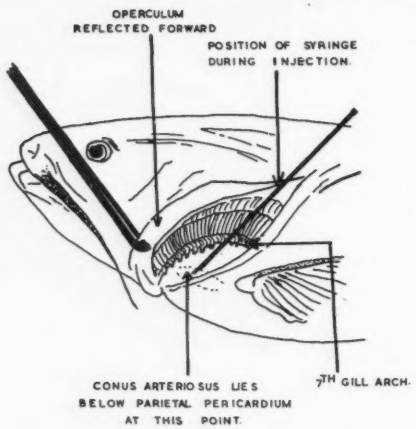

FIG. 1. Injection route.

tissue analysis. The alloxan dose used, 400 mg./kg., produced hyperglycemia in 92% of the experimental fish. At regular intervals, from 3 to 96 hours after treatment, as indicated in Table 1, blood samples were drawn from the conus arteriosus, as suggested by Simpson (19), of the treated and control fish. Immediately after blood sampling, randomly selected control and experimental fish were sacrificed by concussion and islets were removed for histological examination. Blood glucose was measured by a Folin-Malmros micro method, modified for fish blood (10). After fixation in Bouin's fluid, de-

TABLE 1. BLOOD SUGAR* AND BETA CELL† CHANGES AFTER ALLOXAN

Hours after alloxan	Mean* blood glucose, mg.%	Beta granulation†	Other histological features
3	121 ± 28	4-II 1-III	None
6	168 ± 30	1-II 3-III	Hydropic degeneration in 1; pycnotic nuclei in 2
9	124 ± 29	5-III	Pycnotic nuclei and peripheral granulation in 1
12	151 ± 25	1-II 4-III	Slight cytoplasmic disintegration; pycnosis in 2
24	169 ± 36	1-II 4-III	Cytoplasmic degeneration in 4
48	140 ± 12	1-II 1-III 2-IV	pycnotic nuclei in 4, hydropic degeneration in 2
72	157 ± 20	2-II 2-III	Pycnosis in 3; Extensive cytoplasmic degeneration
96	150 ± 15	2-III 2-IV	Cytoplasmic and nuclear disintegration in 2; many pycnotic nuclei in 2

* Normal mean blood sugar—59 mg.% ± 19.
† Arabic numerals in granulation column indicate number of fish observed; Roman numeral indicates degree of granulation, from I (normal) to IV (total degranulation).

hydration in ethyl cellosolve and embedding in 60° Tissuemat, islets were sectioned at 5μ. Sections were stained by Scott's (17) variant of Gomori's aldehyde-fuchsin, with phloxine and fast green FCF counterstains, and by 2 alpha cell methods (1). To avoid errors from possible chance variation in staining, slides were stained in randomized groups, each of which included control and experimental tissues. Islets of 79 fish, 43 control and 36 alloxanized, were classified on an arbitrary scale of beta granulation, from I (normal granulation) to IV (total degranulation). Using another group of 20 fish, we followed blood sugar levels for 14 days, without histological study.

RESULTS AND DISCUSSION

Treated fish differed from control animals in blood sugar level and pancreatic histology. The morphological and blood glucose changes were closely related in time and were progressive through the period studied.

In the intact fish, the mean value for blood glucose was 59 mg.%, with a standard deviation of 19 mg.%. This value was calculated from 237 determinations on 100 normal fish. The total range encountered was 10–120 mg.%. As is shown in Figure 2, extreme values were infrequent.

Alloxanized fish reached blood sugar levels as high as 200 mg.%, (the upper limit of the method) in 179 determinations on 50 animals. The mean value for the experimental group was 121 mg.% 3 hours after treatment, more than twice the mean of controls. In Figure 3 are shown the progress of the hyperglycemia and the comparable control levels. Six hours after treatment, the experimental mean was 170 mg.%, nearly 3 times the normal level. A minor drop, at 9 hours, is not statistically significant. Beyond this period, hyperglycemia persisted for 14 days, when the experiment was ended.

Both in magnitude and variability, the values found resemble those reported by Simpson (19) for catfish, and by others (7, 11, 16) for other fish.

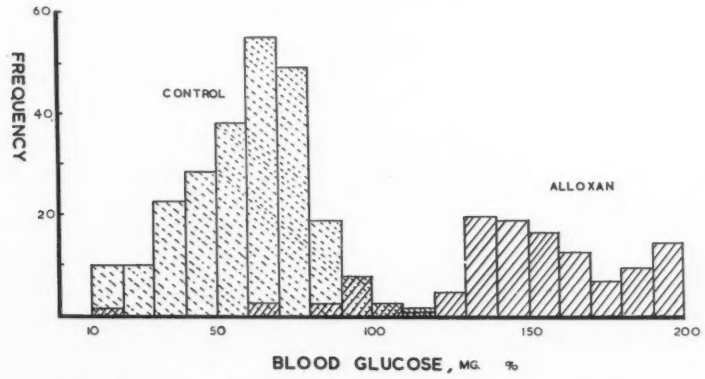

FIG. 2. The distribution of blood sugar levels in control and experimental catfish. The frequency shown at the high end of the blood glucose scale may reflect the 200 mg.% upper limit of the blood glucose method used. Values above 200 mg.% would be read as 200 mg.%.

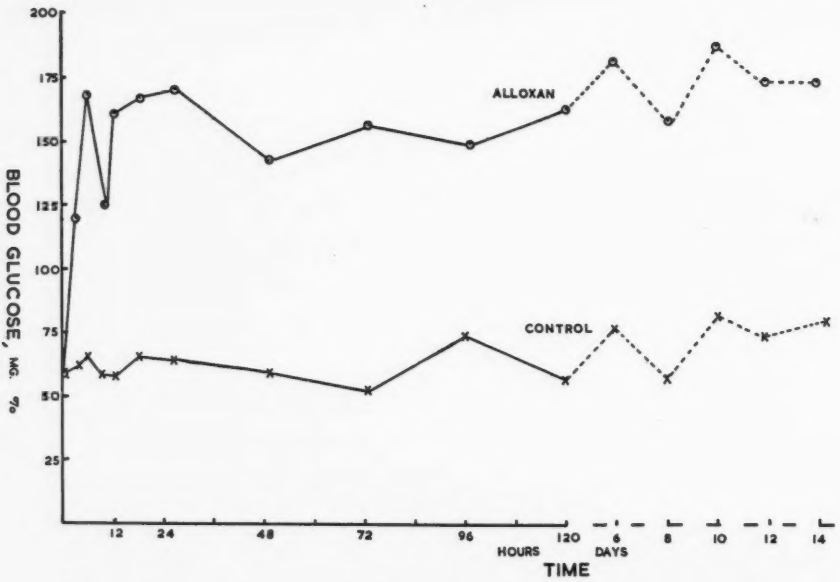

FIG. 3. Effect of alloxan on catfish blood glucose. Broken lines beyond 120 hours indicate reduction of number of fish sampled at each period.

No indication was found of the hypoglycemia observed by Saviano (16), nor was so large a proportion of the treated fish resistant to alloxan. The method used precluded observation of the extreme hyperglycemia found by Saviano in *Scorpaena*, but the frequency distribution plotted in Figure 2 does not suggest that such high values could have been common. The eight-fold increase reported for *Scorpaena* appears unusual for fish or mammals, although comparable to that of steroid-treated fowl. (13).

While the blood sugar changes of the catfish have been found similar to those reported by Saviano, the normal histology and the histological response to alloxan have been found markedly different. The principal islet of the catfish is a nodule, 2–3 mm. in diameter, suspended in the mesentery near the anterior third of the splenic artery. Along the course of this artery are distributed 40–50 minor islets. In islets of both sizes, the richly vascularized endocrine tissue is accompanied by variable, but often extensive, endocrine tissue, as shown in Figure 4. The beta cells form the most common endocrine element, uniformly distributed among alpha cells and a third type of cell, without the localizations described for other fish by Grosso (3) and Saviano (16).

In the normal beta cell, most of the cytoplasm is filled with the characteristic granules, stained intensely with aldehyde-fuchsin, as shown in Figure 5. Striking departures from the normal morphology were found

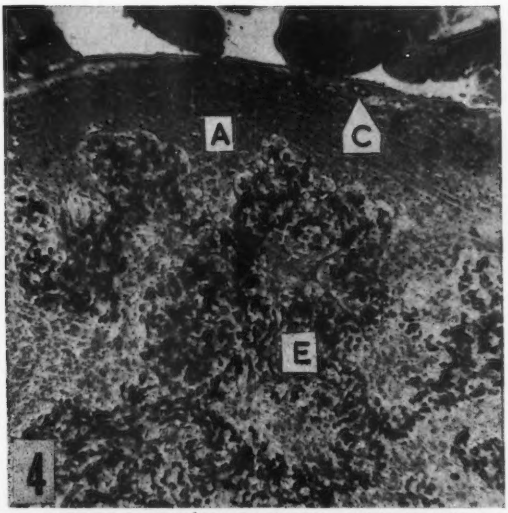

FIG. 4. Normal catfish pancreas, with exocrine (A), endocrine tissue (E) and connective tissue capsule (C). Aldehyde-fuchsin, ×150.

FIG. 5. Normal endocrine pancreas of catfish with beta granulation (B). Alpha and type 3 cells are intermixed among beta cells. Aldehyde-fuchsin, ×1500.

in alloxanized animals. Degranulation was apparent within 3 hours of treatment and progressed through the period studied, as shown in Figure 5 and Table 1. Six hours after treatment, nuclear and cytoplasmic degeneration were apparent, and became more severe in later samples. The con-

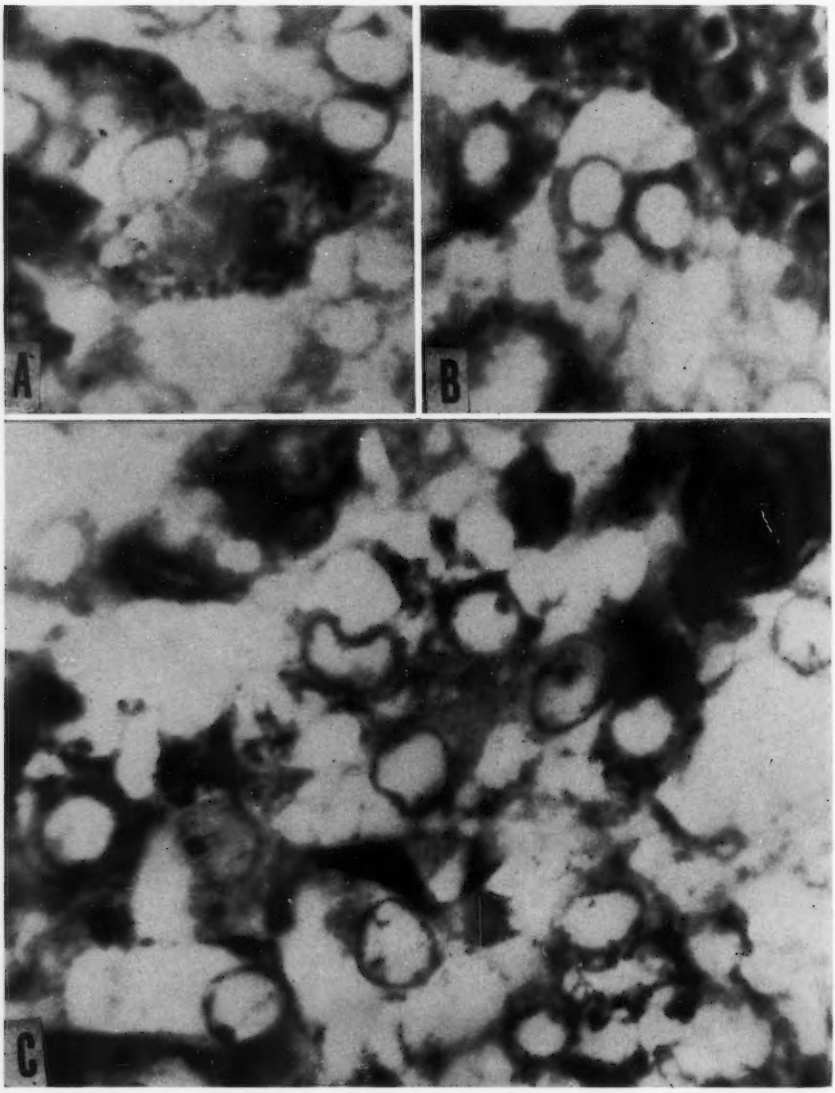

FIG. 6. Pancreatic changes after alloxan. (A) Stage II degranulation. (B) Stage III degranulation. (C) Stage IV degranulation, with nuclear and cytoplasmic damage. Aldehyde-fuchsin, ×1500.

tinuation of this trend through 96 hours suggests an alloxan reaction more prolonged than that of mammals (9, 12). No definite mitotic figures or signs of beta cell recovery were seen, although pairs of cells with small nuclei, suggestive of daughter cells of a recent division, were found in some of the late samples and were never seen in intact animals or early samples.

The tissues studied provided no support for Doerr's explanation (2) of the alloxan resistance of his carp and tench in terms of an extensive supply of immature beta cells available for replacement of those damaged by the drug. The development, in the catfish, of complete degranulation is equally contrary to the observations of Pallot and Schatzle (14), who have described similar reserves of cyprinid fishes. The prominent alpha cell lesions reported by Saviano and the marked zymogen cell damage seen by Saviano and by Pallot and Schatzle were not apparent in the catfish.

The absence of all these features indicates that the histological response of the catfish resembles that of the guppy (3) and of the common laboratory mammals, and differs very markedly from that of other fish, including *Opsanus*,[3] *Scyllium*, *Motella*, *Scorpaena*, carp and tench. The clarification of this inherent difference, in this particularly favorable experimental material, should advance understanding of the mechanism of the diabetogenic action of alloxan. The suggestion of Doerr and of Pallot and Schatzle, attributing the alloxan resistance of certain fish to cell reserves and cell transformations, similar to those described in the rat by Hughes (5) and in the guinea pig by Johnson (6), Hausberger and Ramsey (4) and Woerner (20), indicates that comparative studies among fish may be fruitful of understanding of beta cell regeneration and the normal controls of a functional beta cell population.

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[3] Current study of *Opsanus* has demonstrated zymogen cell lesions in this animal, with transitory beta cell destruction and hyperglycemia. Details will be reported shortly.

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BLOOD SUGAR STUDIES IN THE BULLFROG, *RANA CATESBIANA*[1]

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ABSTRACT

Over a period of 2½ years, blood glucose values of 154 normal bullfrogs averaged 13.51 mg.% with 28% showing complete sugar absence. No correlation could be found between blood glucose level and apparent nutritional state of the animal or season of the year. Among factors which induced hyperglycemia were repeated removal of blood samples (without albumin restitution), intra-arterial glucagon >epinephrine, and extracts of bullfrog, angler fish, and dogfish gastrointestinal tissues presumed to contain glucagon. Alloxan, beef STH, and bullfrog pituitary extract failed to raise the blood sugar level significantly. Bullfrogs were somewhat less tolerant of intra-arterially injected monosaccharides than rabbits and responded slowly to exogenous insulin. A brief discussion follows on the relationship of these data to the apparently normal maintenance of an hypoglycemic state in bullfrogs.

IN RECENT studies the author has reported either complete absence of blood glucose (1) or very low sugar values (2) for normal bullfrogs. Wearn and Richards (3) noted absence of blood sugar in several specimens of *Rana pipiens*, attributing their observations to the fact that the frogs had been starved. Apparently little attention was paid to their data since there has never appeared, to my knowledge, any published confirmation or disagreement. Giese (4) reported that activity of laboratory frogs in winter could be improved by glucose injection, but did not indicate that any actual blood sugar values were obtained. Related species, *i.e.*, *Bufo arenarum* (5, 6), *Bufo vulgaris*, *Rana temporaria* and *Rana esculenta* (7), at least when tested by two other standard procedures, regularly show substantial amounts of blood glucose, and no instance of sugar absence has been recorded for any other vertebrate (8).

The discovery of active vertebrates with little or no blood sugar naturally prompted us to look into a variety of factors which might account, separately or in combination, for the anomalous values obtained. The ex-

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periments reported here were designed in the hope of finding some key to the seeming enigma of bullfrog blood glucose.

MATERIALS AND METHODS

Frogs, with a weight range of 80 to 700 gms., were purchased from collectors in Louisiana and Wisconsin except for several specimens found locally in the vicinity of Woods Hole during the summer months. After arrival the animals were stored in granite laundry trays in shallow running water so that their body temperatures were maintained between 16° and 20° C. Because the frogs were used within ten days, no attempt was made to feed them except where specifically mentioned below.

Blood samples were always taken from the conus arteriosus after posteriorly pithing the frog, exposing the pericardial area, and routine heparinization (5 mg./kg.). Since repeated samples from the same frog were often desired, a method was devised whereby a #22 hypodermic needle was left inserted in the right ventral aorta throughout the experiment. All injections were made and all blood removed via this route simply by attaching and detaching various syringe barrels to the stationary needle. Any other method which involved multiple arterial punctures invariably resulted in large blood losses. Some experiments consumed as much as four hours with good circulation maintained throughout; most experimental procedures were completed in less than two hours.

Blood sugar levels were determined according to the method of Nelson (9), except that 0.04N NaOH was substituted for distilled water and $Ba(OH)_2$ in making protein-free filtrates. Duplicate filtrates were made on several occasions with H_2SO_4 and sodium tungstate, but these gave identical sugar values to those made with NaOH and $ZnSO_4$. It should be pointed out that the Nelson technique, because of its greater specificity, gives sugar values 10–15% lower than most other cupric reduction procedures.

EXPERIMENTAL PROCEDURES AND RESULTS

Normal blood sugar values for some 154 animals over the $2\frac{1}{2}$ years of this study gave an average reading of 13.51 mg.%. Distribution of these values over the year (Fig. 1) indicates a tendency for lower glucose readings during the months that northern animals customarily spend in hibernation. Since more than two-thirds of the frogs in this study came from Louisiana where the species is active throughout the year, it is not likely that the preponderance of low glucose values could be attributed entirely to the hibernation state. The general year-around occurrence of instances of glucose absence tends also to indicate against activity as the controlling factor.

A large majority of the frogs was autopsied in an effort to correlate presence of food in the stomach with blood glucose level. No relationship could be discovered, however, since many animals whose stomachs were distended with digesting crayfish had no detectable blood glucose, whereas many frogs with empty stomachs had some of the higher sugar values obtained. A force-feeding experiment was carried out on six frogs by daily rations of beef liver for a period of two weeks (February). Blood glucose on the fifteenth day showed four animals with sugar absence and two with only 5 mg.%. Four animals which received 100 mg. of glucose (5% aqueous solution) intraperitoneally twice daily for four weeks, and twice

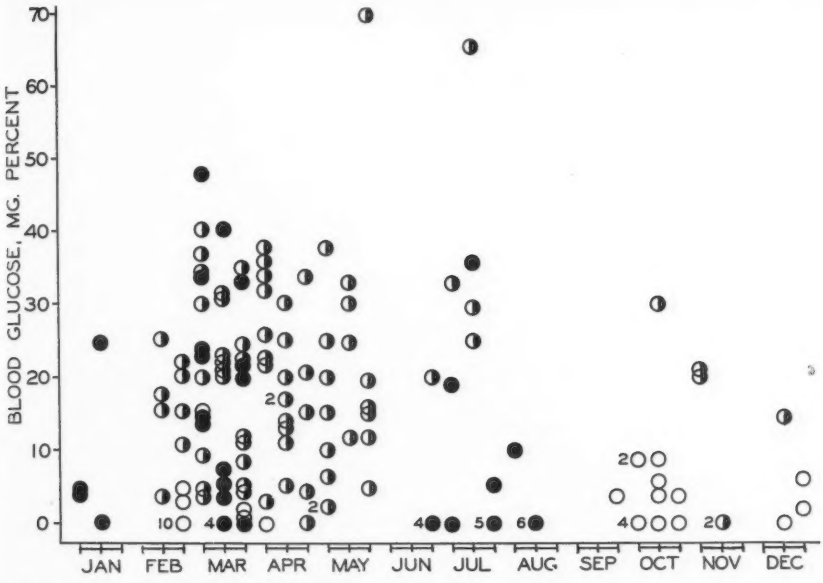

FIG. 1. Blood glucose values for 154 intact bullfrogs as distributed over the year. For convenience months have been divided into thirds, *viz.*, 1st to 10th, 11th to 20th, and 21st to 31st. Open circles, 1956; half-closed circles, 1957; closed circles, 1958.

weekly feedings of beef liver, all gave zero glucose values when blood was taken 16 hours following the last glucose injection.

Among experimental procedures which resulted in some amount of hyperglycemia, a reduction in blood volume (and, consequently, blood pressure) by hemorrhage or repeated removal of blood samples was of primary importance in interpreting early experimental data. Figure 2 shows the progressive hyperglycemic effect of taking numerous blood samples and the correction of this difficulty by injection of an equal volume of 3% human blood albumin in Ringer's solution following removal of each blood sample. Fortunately, the hypergylcemic effect of reduced blood volume was discovered early in experimentation so that routine replacement of blood with albumin was an integral part of all experiments reported below. It is assumed without any supporting evidence that hyperglycemia following a drop in blood volume and blood pressure comes about reflexly, as in mammals, through the endogenous release of epinephrine or through general sympathetic stimulation.

Response of bullfrogs to glucagon injection is dramatic, but is probably more closely related to some uncontrollable factor such as the indefinable nutritional state of the animal than to the dosage of hormone administered (Table 1). The sharpest rise in blood sugar level occurs within the

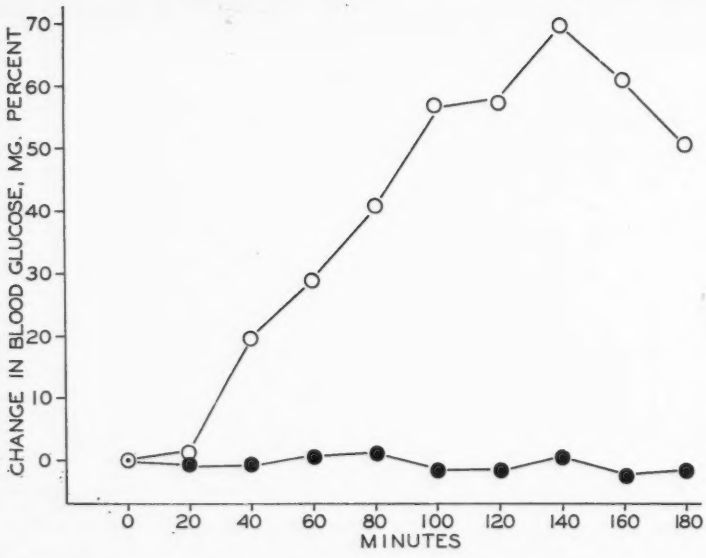

FIG. 2. Spontaneous development of hyperglycemia due to taking of repeated blood samples every 20 min. (open circles). Maintenance of nearly constant blood glucose level when blood as removed is replaced at once with equal aliquots of 3% blood albumin in Ringer's solution (closed circles). March.

first 20 minutes after glucagon injection, the peak response ordinarily occurring, with amorphous glucagon, within the first 40 minutes. Crystalline glucagon seemed to produce somewhat lower levels of hyperglycemia during the first 20 minutes, but the effect was a progressive one up to at least 90 minutes after injection. In contrast to these data, epinephrine injection (10) raised the blood sugar level by much smaller amounts (av. 32 mg.%, max. 45 mg.%).

In view of the difference in degree of response to epinephrine and glucagon, it seemed interesting to investigate the effects of a second dosage of either epinephrine or glucagon after an original administration of one of the hormones had had ample opportunity to act. Results (Table 2) showed that even though a moderate dosage of glucagon had been allowed to exert its effect, a second dosage produced a still greater degree of hyperglycemia. If glucagon was administered 40 minutes following a dosage of epinephrine, a second, more marked, wave of hyperglycemia occurred. If, on the other hand, epinephrine was injected 40 minutes after administration of glucagon, no noticeable hyperglycemic response was produced by epinephrine.

Alloxanization (up to 800 mg./kg. intraperitoneally) failed to induce any hyperglycemia nor any detectable islet damage upon microscopic examination of Gomori-stained tissue. Animals seemed to tolerate the

TABLE 1. RESPONSE OF BULLFROGS TO INTRA-ARTERIAL INJECTION OF GLUCAGON*

Date	Glucagon	Dosage, μg./kg.	Blood sugar, mg.% at				
			0 min. (initial)	20 min.	40 min.	60 min.	90 min.
3-19	amorph.	10	0	4	0		2
7-15		10	25	24	22	27	
4- 2		25	0	112			
5-20		30	25	94	88	107	141
4- 3		40	36	98	103		138
5-17		50	12	47	77	62	
5-17		100	30	81	88	79	106
5- 5		150	10	96	125	107	121
5-21		200	15	60	42		44
5-24		200	70	170	209	252	206
1-15	cryst.	10	0	0	5	0	4
1-15		25	25	83	135	141	
1- 9		50	4	54	63	75	
7-15		100	36	65	69	86	93
7-10		200	19	33	37	41	50

* Dr. W. R. Kirtley, Lilly Laboratories, gratefully supplied the glucagon used. Amorphous glucagon was from batch #258-234B-40 and crystalline glucagon from lot #258-234B-54-2. Both were used as a 0.02% solution in glycine buffer, pH 9.4, stored in a refrigerator.

alloxan well, as there was no mortality among six animals for ten days following injection of the drug. Attempts to induce the diabetic state by total or subtotal pancreatectomy were unsuccessful, for no frog survived longer than an hour or two following the operation. A large portion of the frog pancreas surrounds the common bile duct and a portion of the the hepatic portal vein, making even subtotal pancreatectomy extremely difficult.

TABLE 2. RESPONSE OF BULLFROGS TO REPEATED DOSAGES OF HYPERGLYCEMIC AGENTS

Date	Original agent inj. at 0 min.	Blood sugar, mg.% at			Second agent inj. at 40 min.	Blood sugar at	
		0 min. (initial)	20 min.	40 min.		60 min.	80 min.
4-26	AG*	34	74	87	AG	116	201
5- 1	AG	25	45	40	AG	75	134
5- 3	AG	3	59	50	AG	71	100
4-29	AdCl†	0	5	19	AG	73	127
5- 8	AdCl	15	20	30	AG	112	118
5-10	AG	3	74	85	AdCl	70	80
5-10	AG	38	92	102	AdCl	83	82

* Amorphous glucagon, 100 μg./kg.
† Adrenalin chloride, Parke, Davis Co., 100 μg./kg.

Administration of beef STH[3] also failed to induce hyperglycemia both when given in twice-daily intraperitoneal injections (2.5 mg. each) for two days prior to pithing and when given, like glucagon, intra-arterially (5 mg.) following pithing. Injection of crude bullfrog anterior lobe extract (Ringer)

[3] Supplied by Dr. Sanford L. Steelman, Armour Laboratories.

induced a very small (10–15 mg.%) rise in blood sugar 40 minutes after injection. Nothing is known of the STH content, if any, of such a preparation. Four bullfrogs, hypophysectomized from 3 to 7 days previously, had no detectable blood glucose.

Extracts of gastro-intestinal tissues of the bullfrog, prepared according to the method of Sutherland and de Duve (11), showed the presence of some moderately potent hyperglycemic agent presumed to be glucagon in pancreas > gastric mucosa > duodenum. These preparations were incubated for 4 hours in 0.1% cysteine to destroy any possible traces of epinephrine or its analogues. Blood glucose rises of from 20 to 65 mg.% were achieved within 40 minutes after intra-arterial injection of these extracts, dosages (0.5 to 1.0 cc. in volume) being equivalent to 0.25 to 1.0 gm. of fresh tissue. Injection of these same dosages into rabbits failed to induce any hyperglycemia, suggesting that amphibian glucagon, if present in these extracts, differs significantly from mammalian glucagon.

Injection of 0.5 cc. extract (representing 0.4 gm. fresh tissue) of the principal islet of the angler, *Lophius piscatorius*, induced a 41 mg.% rise in frog blood glucose in 40 minutes. There was a 46 mg.% rise in blood sugar of a bullfrog 40 minutes after injection of 1.0 cc. extract (1 gm. fresh tissue) of dogfish, *Mustelus canis*, pancreas. Extracts of bullfrog pectoralis muscle prepared in the same way as the above tissues, induced some hyperglycemia (as much as 50 mg.% in 40 minutes) before incubation with cysteine, but this potency was completely lost after cysteine treatment.

Sugar tolerance tests on bullfrogs showed them to compare favorably with mammals in ability to bind injected monosaccharides and even maltose. Injection of Evans blue and subsequent colorimetric analysis showed the blood volume to be 6.5 to 7% of body weight. On this basis bullfrogs convert 52% of glucose (4 cases), 51% of galactose (3 cases), 49% of fructose (4 cases), 45% of arabinose (2 cases), and 38% of mannose and maltose (2 cases each) within five minutes after intra-arterial injection, each sugar dosage having been calculated to raise the blood sugar level by 100 mg.%. The relatively slow hypoglycemic action of insulin is well demonstrated when the hormone is used to modify the tolerance curves of glucose and fructose (Table 3).

Simultaneous injection of monosaccharide along with crystalline glucagon gave interesting results (Table 3) in that injected sugar seemed to inhibit the continuing hyperglycemic action of glucagon. Equally interesting is the observation that glucagon injection did not inhibit the usual amount of glycogenesis which normally followed injection of these sugars.

DISCUSSION

This paper has not attempted to deal with perhaps the most intriguing problem presented by bullfrogs, *i.e.*, how can such a vertebrate manage to survive, or indeed show appreciable activity, with blood glucose unavail-

TABLE 3. EFFECT OF INSULIN AND GLUCAGON ON THE MONOSACCHARIDE TOLERANCE CURVES OF BULLFROGS

Date	Sugar*	Hormone†	Blood sugar, mg.% at				
			0 min. (initial)	5 min.	30 min.	60 min.	90 min.
3- 6	Fructose	None	48	84	94	79	74
3- 8	Glucose	None	33	80	86	72	70
3- 5	Fructose	4 U. Insulin	34	107	90	63	48
3-25	Fructose	8 U. Insulin	0	54	27	22	38
2-22	Glucose	4 U. Insulin	20	53	43	36	26
3- 1	Glucose	4 U. Insulin	9	68	44	32	25
3-20	Glucose	8 U. Insulin	0	59	44	37	33
3-26	Fructose	Glucagon	1	47	26	38	38
3-26	Glucose	Glucagon	5	55	32	35	35

* Sugar dosages were calculated to raise the blood sugar level by 100 mg.% on the basis of body weight and were administered in 0.1% saline solution immediately after initial blood sample.

† Insulin or glucagon was given intra-arterially 10 minutes after sugar. Insulin was Iletin Lilly (40 units per cc.) and glucagon was crystalline in a dosage of 50 μg./kg.

able or in extremely short supply? The fact that more than 25% of animals tested showed absence of glucose indicates that the whole species must be subject at one time or another to lack of blood carbohydrate. It may be convenient to say that frog tissues have become accustomed to doing without ready sugar, but to make this statement in no way answers the question of how cells have modified their metabolism to meet this unusual circumstance. The answer to this phase of the problem must be provided by biochemical studies at the level of blood-tissue interrelationships. Any suggestions presented at this time would be merely guesses for lack of information; nevertheless, the subject is a tempting one for much speculation.

As to explanations for the maintenance of blood glucose at such low levels, the results presented in this communication have not appeared to answer that phase of the problem, either. Except possibly for its negative response to STH and the minimal response to pituitary extracts, the frog seems to react to experimental procedures in virtually the same manner as higher vertebrates which customarily maintain considerably higher blood glucose levels. Frogs were even less tolerant of injected sugar than rabbits, which in our laboratory converted an average of 75% of glucose within five minutes after intravenous injection, as opposed to the frog's 50% or so. This response indicates that frogs perhaps have a relatively lower level of circulating insulin, and therefore have the capacity for maintaining a higher level of blood glucose than do rabbits. It may also be inferred from the results of monosaccharide injection that the appearance of sugar in the blood stream does not induce a surge of endogenous insulin secretion which, if prolonged, might account for the maintenance of low sugar

levels. The slow response to exogenous insulin indicates a relative insensitivity of frog tissues to this hormone, and would tend, if anything, to allow hyperglycemia rather than the severe hypoglycemic condition encountered.

Responses to glucagon put the frog in the mammalian category, apart from the only other amphibian whose reaction to glucagon has been tested. Miller and Wurster (12) found that glucagon did not raise blood sugar in *Taricha torosa*. This salamander also has normally low blood glucose (15 to 20 mg.%), but not glucose absence, and does not appear to have alpha cells within its pancreatic islets. Bullfrogs definitely have islet cells which give the customary reactions of alpha cells when stained with Gomori's chrome hematoxylin and phloxin or with aldehyde fuchsin and trichrome (author's unpublished study).

The failure of bullfrogs to respond to beef STH and the weak response to a frog pituitary extract should have further investigation. It would also be helpful, indeed, to know the response to STH as isolated from fish, or better still, frog, pituitary glands, for it is very possible that the frog is insensitive to this pituitary factor as derived from a mammal. If STH from lower forms failed to induce hyperglycemia in frogs, it is entirely possible that an explanation for the widespread hypoglycemia could lie in that relationship. Even though glucagon appears to be present in frog gastro-intestinal tissues, it may not function in the absence of a factor to effect its release. In that event, levels of insulin, however high or low, would then be unopposed and could account for the normally hypoglycemic state.

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STUDIES ON THE MECHANISM OF HYPOTHALAMIC CONTROL OF THYROTROPIN SECRETION: EFFECT OF THYROXINE INJECTION INTO THE HYPOTHALAMUS OR THE PITUITARY ON THYROID HORMONE RELEASE[1]

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ABSTRACT

In an attempt to ascertain whether or not thyroxine can change hypothalamic as well as pituitary activity in regulating thyrotropin secretion, microinjections of thyroxine were made directly into a localized area of the hypothalamus or into the anterior pituitary. Determination of thyroid secretion was utilized in evaluating possible changes in thyrotropin secretion. Inhibition of thyrotropin secretion was produced by injection of thyroxine into either the "thyrotropin area" of the hypothalamus or into the anterior pituitary. Injection into the subarachnoid space or into other areas of the hypothalamus was without effect. Hypothalamic injections had a latent period of six to nine hours before thyrotropin secretion was inhibited, whereas the inhibition was immediate after pituitary injections. The duration of inhibition was approximately 20 hours in either location. It is suggested that two separate mechanisms might control thyrotropin secretion, a slower one acting through the central nervous system and a direct one in the anterior pituitary itself.

MANY workers have studied the effect of hypothalamic lesions, pituitary stalk section or electrical stimulation of the hypothalamus on thyroid function. Recent studies by Greer (1, 2) have demonstrated that destruction of the anterior hypothalamus of the rat prevents the thyroid hypertrophy which normally follows propylthiouracil treatment. This has been confirmed by Bogdanove and Halmi (3) and others (4, 5). It thus seems well established that the hypothalamus plays an important role in the control of thyrotropin secretion by the pituitary.

It has been postulated that thyroid hormone controls thyrotropin secretion by altering the activity of the hypothalamus and thereby the pituitary (6). Such a hypothesis is attractive, but there is no actual proof of this mechanism and the primary role of the hypothalamus is still uncertain.

In the present study, in an attempt to ascertain whether or not thyroxine can change hypothalamic as well as pituitary activity in regulating thyrotropin secretion, microinjections of thyroxine were made directly into a localized area of the hypothalamus or into the anterior pituitary. Deter-

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mination of thyroid secretion was utilized in evaluating possible changes in thyrotropin secretion.

MATERIALS AND METHODS

Forty seven adult male Holtzman rats, weighing 212 to 344 gm., were used. Animals were fed a Remington low iodine diet[2] for at least 7 days in order to get a high uptake of I^{131} by the thyroid gland. Animals used in the hypothalamic injection experiments were divided into three groups: Posterior, Anterior and Pre-optic. The number of experimental and control animals in each group is shown in Table 1.

TABLE 1. SUMMARY OF THE RESULTS OF SALINE AND THYROXINE INJECTION INTO THE HYPOTHALMUS OR PITUITARY ON THYROIDAL SECRETION

	Saline controls (No. of animals)	Thyroxine injection			Inhibition	
		No. of animals	Exhibiting depressed thyroid secretion		Onset aft. T4 inj. (hrs.)	Duration (hrs.)
			(Number)	(Per cent)		
Hypothalamus Group						
I. Post.	2	3	1	33	12	16
II. Ant.	7	18	16	89	8.5 ± 1.2[1]	20.6 ± 1.1
III. Pre. Op.	—	2	0	0	—	—
Pituitary Group						
Pituitary	3	4	4	100	<0.5	20.6 ± 6
Subarachnoid space	2	2	0	0	—	—

[1] Mean and S.E.

Number 27 hypodermic needles, 0.3 mm. in external diameter, were inserted in the midline with a stereotaxic instrument (11) employing the following parameters for each group: Posterior, 4.5 mm. anterior to the ear bar, 8.5 mm. deep (from the top of the skull); Anterior, 6.5–7mm. anterior, 8–8.5 mm. deep; Pre-optic, 8 mm. anterior, 8 mm. deep. In the Pituitary group, the needles were inserted in the anterior lobe. After insertion, the needle was fixed to the skull by four watch case screws (Fig. 1).

l-Thyroxine in propylene glycol[3] was diluted with physiological saline to a concentration of 0.25 μg. thyroxine /0.02 ml. saline.

Twenty-four hours after the intraperitoneal administration of 20 μc. of I^{131}, 30 mg. of propylthiouracil in 10% gum acacia solution was given subcutaneously daily at rotated sites to block the reaccumulation in the thyroid of I^{131} liberated from degradation of endogenously labeled thyroxine. Counts over the thyroid region were made five times daily under light ether anesthesia by means of an externally placed scintillation counter and each point was expressed as a per cent of initial (24 hour) thyroid uptake and plotted on semi-log paper.

After obtaining a straight line of more than five individual points, 0.25 μg. *l*-thyroxine contained in 0.02 ml. saline was injected into the hypothalamus, pituitary or subarachnoid space through the needles previously fixed to the skull. In the control animals of each group, 0.02 ml. saline were injected in the same way. The "dead space" of each needle was taken into account when calculating the volume injected. This averaged 0.02 ml.

[2] Iodine Deficient Test Diet supplied by General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Kindly supplied by Dr. A. E. Heming, Smith, Kline & French Laboratories.

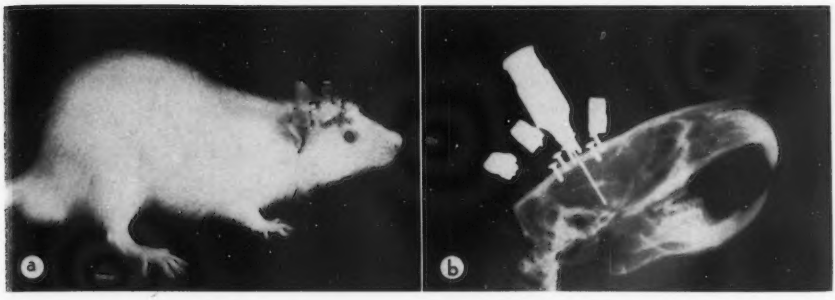

FIG. 1. Needle inserted into the anterior hypothalamus of a rat. (a) Photograph of rat with needle in place; (b) Radiograph of skull with fixed needle.

In the animals in which a possible stress effect of the microinjection was studied, 0.02 ml. physiological saline was injected into the anterior hypothalamus or the pituitary one hour after I^{131} injection, and the 24 hour uptake by the thyroid gland was measured by *in vivo* counting.

The exact position of the needles, cytological reaction around the needles, lesions due to the needles and possible infection were studied in 21 of the 29 thyroxine-injected animals in serial histological sections cut at 10 μ and stained with hematoxylin-eosin.

RESULTS

Thyroid hormone release in the non-operated control group was measured for about 100 hours. The plots obtained in four animals were exponential throughout the period of the experiment, although several points were rather irregular.

Before starting the microinjection experiment using thyroxine, the effect of systemic injection of thyroxine (0.25 μg.) and the possible non-specific effect of microinjection into the hypothalamus and the pituitary were studied. None of the animals which received a systemic injection of 0.25 μg. thyroxine showed a change in the release slope (Fig. 2). However, complete plateauing of the slope or slowing of the rate persisted for about 19 hours in animals that received systemic injections of 5 μg. thyroxine. Onset of inhibition seemed to be within four hours in all affected animals.

The effect of microinjection of saline into the hypothalamus or the pituitary on thyroidal uptake of I^{131} is shown in Fig. 3. The 24-hour uptake was 20.8%, 18.7% and 19.9% respectively in the Anterior (hypothalamic) injection group, the Pituitary group and the untreated control group. Statistically there was no significant difference between them.

1. *Effect of Hypothalamic Injection of Thyroxine on Thyroid Hormone Secretion*

In the Posterior group, a change of slope was observed in one of three experimental animals. Twelve hours after injection, thyroidal secretion

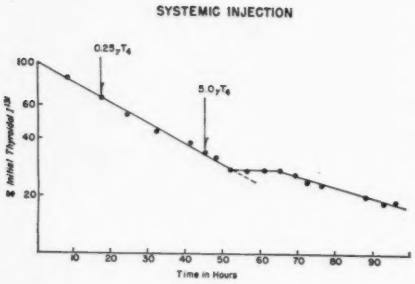

FIG. 2. The effect of systemic injection of 0.25 μg. *l*-thyroxine and subsequent systemic injection of 5 μg. *l*-thyroxine on thyroid hormone secretion. After 0.25 μg. no change was produced, but after 5 μg. transient complete inhibition occurred.

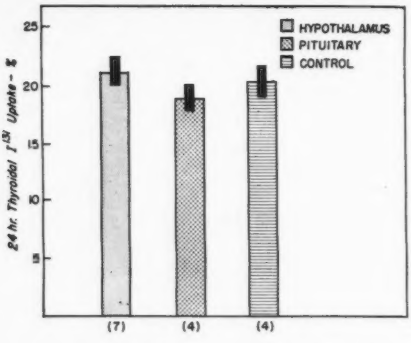

FIG. 3. Effect of saline injection into the anterior hypothalamus and the pituitary on thyroidal radioiodine uptake. 0.02 ml. saline was injected one hour after I^{131} injection in the hypothalamus and pituitary group. Number of animals is indicated in parentheses.

was completely inhibited. This inhibition persisted for 16 hours.

In the Anterior group, a change in rate occurred in 16 of 18 animals. The time of onset varied from animal to animal, but most striking was the fact that inhibition occurred 2 to 16 hours, usually 6 to 9 hours, after thyroxine injection (average 8.5 hours), while in only two animals did a change in slope occur immediately. The duration of inhibition also varied from animals to animal, but there was less fluctuation than with time of onset. The average length of inhibition was 20.6 hours. In most of the animals, complete flattening occurred (Fig. 4), but in some animals only a change of slope was observed (Fig. 5). It was difficult to detect any relationship between the onset of change, degree of change, or its duration.

In the Pre-optic group, none of the experimental animals showed a decreased thyroidal secretion after thyroxine injection. In no instance did injection of saline change the rate of loss of I^{131} from the thyroid.

Thyroxine was injected twice in five animals, three of the Anterior group and two of the Posterior group. A change in thyroidal secretion rate occurred both times in the Anterior animals. The onset of change and the duration were in good agreement in all three rats (Fig. 6). In the Posterior animals no change was produced by either injection of thyroxine (Fig. 7).

II. *Effect of Injection of Thyroxine into the Pituitary on Thyroid Hormone Secretion*

In the Pituitary group, it was observed that a change of slope occurred immediately after thyroxine injection in all four animals, whereas there

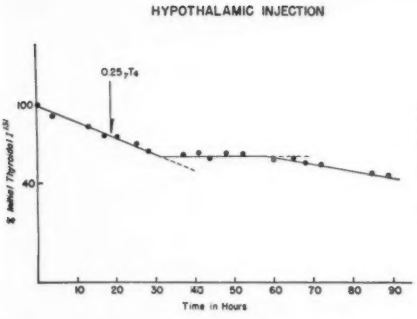

FIG. 4. Ten hours after injection of 0.25 μg. *l*-thyroxine into the anterior hypothalamus, complete inhibition of thyroidal secretion occurred and lasted approximately 30 hours.

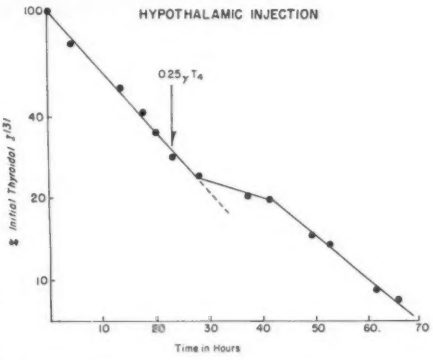

FIG. 5. Five hours after injection of 0.25 μg. *l*-thyroxine into the anterior hypothalamus, a change of slope occurred which lasted about 14 hours.

was no effect from saline injection into the pituitary nor from thyroxine introduced into the subarachnoidal space under the pituitary. In two of four experimental animals the slope became completely flat (Fig. 8), while in another two animals only a slowing of the rate occurred (Fig. 9). Duration of the inhibition of thyroidal secretion varied from animal to animal as in the hypothalamic experiment, but the average was 20.6 hours, being very close to that produced by hypothalamic injection of thyroxine.

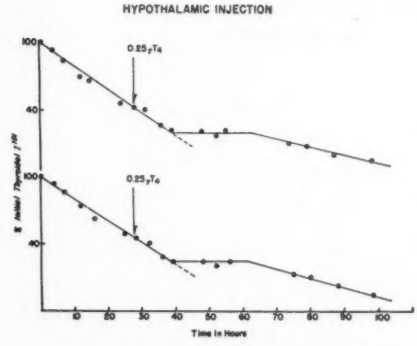

FIG. 6. Repeated experiments in the same animal of the anterior hypothalamus group. The first experiment is above and the second below. In both experiments, inhibition occurred about ten hours after thyroxine injection and lasted for 14 hours.

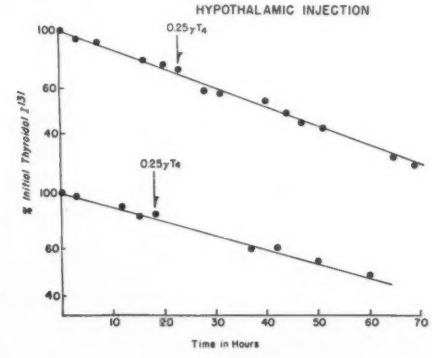

FIG. 7. Repeated experiments in the same animal of the posterior hypothalamus group. In both experiments, no change in thyroidal secretion was produced after thyroxine injection.

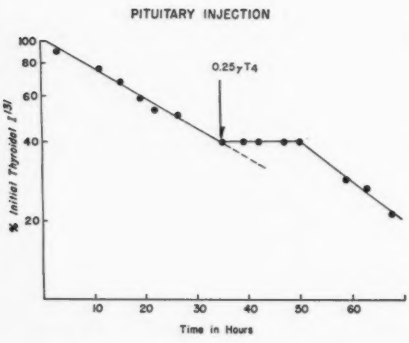

FIG. 8. Soon after the injection of T_4 into the pituitary, complete inhibition lasting 24 hours occurred.

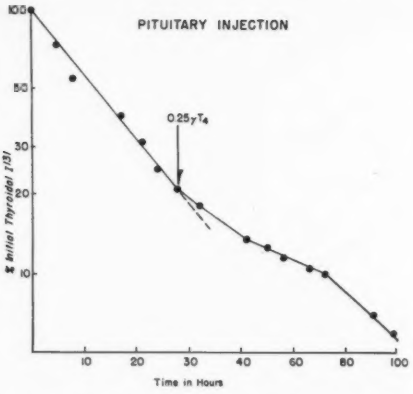

FIG. 9. In this animal, following thyroxine injection into the anterior pituitary, the slope changed but did not completely flatten. This partial inhibition lasted for about 25 hours.

III. *Histological Examinations*

The position where the needles were inserted is shown in Fig. 10. In the Posterior group, the needle in the animal which gave a positive result was situated nearer to the so-called "thyrotropin area" than in the animals which had no change in thyroidal secretion after thyroxine injection.

In the Anterior group, in all animals studied the needles were situated in the thyrotropin area. It was of interest that, in the two animals which gave a negative result, the needles penetrated slightly through the base of the hypothalamus, indicating that the injected thyroxine may not have entered the hypothalamus.

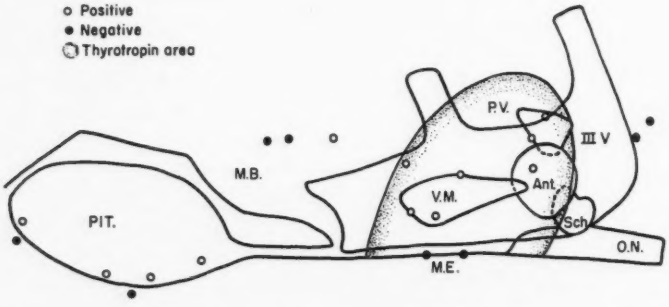

FIG. 10. Diagram of the position of the inserted needles. Solid circles indicate a negative effect and open circles a positive effect. The thyrotropin area is surrounded by a stippled border. P.V. = paraventricular nucleus, III V. = third ventricle, Ant. = anterior hypothalamic area, Sch. N. = suprachiasmatic nucleus, V.M. = ventromedian nucleus, M.E. = median eminence, M.B. = mamillary body, PIT = pituitary.

In the Pre-optic group, the needles were located anterior to the thyrotropin area in both animals. In the Pituitary group, the needles were in the anterior pituitary in all animals; they were just below the pituitary in the Subarachnoidal group.

There was no definite correlation of the anatomical position with the degree or duration of inhibition. Some cytological reaction around the needles was observed, but it was very slight. No lesions or infection due to the needle insertion were found.

DISCUSSION

Previous studies have shown that only lesions in the so called "thyrotropin area" of the hypothalamus, in the midline between the paraventricular nucleus and the median eminence, consistently result in diminution of thyrotropin secretion by the pituitary (7). It has also been shown that the neocortex is not essential to thyrotropin release in the rat (8). Such studies suggest that the hypothalamus may be a primary center controlling thyrotropin secretion from the pituitary although the possibility remains that the hypothalamus is merely an intermediate area transmitting impulses from higher centers to the pituitary.

Von Euler and Holmgren (9) reported that microinjection of systemically ineffective amounts of thyroxine directly into the anterior pituitary would cause a decrease in the release of radioiodine from the rabbit thyroid but that the same amount of thyroxine was without effect when injected into the median eminence or other parts of the hypothalamus. They concluded that the inhibitory effect of thyroxine on thyrotropin secretion was produced only by direct action on the anterior pituitary (9, 10).

In the present study, however, inhibition of thyrotropin secretion was produced by injection of thyroxine into either the "thyrotropin area" of the hypothalamus or into the anterior pituitary. As previously shown by Von Euler and Holmgren (9), injection into the subarachnoid space was ineffective. Thyroxine placed in other areas of the hypothalamus was also without effect. The two animals injected into the "thyrotropin area" in which no inhibition was produced apparently had leakage of the thyroxine into the subarachnoid space, as indicated by the histologic sections of the hypothalamus.

No effect from the injection of similar amounts of saline on either thyroidal secretion or radioiodine uptake was seen.

The data presented here clearly indicate that both hypothalamic and pituitary injection of systemically ineffective amounts of thyroxine can inhibit thyrotropin secretion. Of particular interest is the observation that hypothalamic injection has a latent period of six to nine hours before thyrotropin secretion is inhibited whereas the inhibition is immediate after pituitary injection. The duration of inhibition is approximately the same when thyroxine is injected into either location, being about 20 hours.

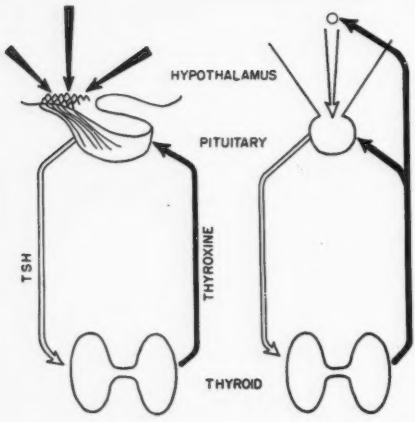

FIG. 11. Diagram of alternate hypotheses to explain the observed inhibitory effect on thyrotropin secretion by hypothalamic thyroxine injection. (1) On the left is shown the possibility of diffusion of injected thyroxine to the origin of the hypophyseal portal vessels and its transport to the anterior pituitary where the inhibitory effect is exerted. (2) On the right is shown a possible negative feed-back mechanism in both the hypothalamo-pituitary-thyroid system and the pituitary-thyroid system.

Two possible explanations of the data presented above can be made (Fig. 11): (1) Thyroxine injected into the anterior hypothalamus takes approximately 8.5 hours to diffuse to the hypophyseal portal vessels and be carried to the anterior pituitary where it exerts an inhibitory effect. (2) Direct inhibition of a primary hypothalamic "thyrotropin center" is produced by thyroxine injection into the anterior hypothalamus. Cells in this area are sensitive to alterations in circulating thyroid hormone concentration but there is a latent period of about 8.5 hours before thyrotropin release by the pituitary is eventually affected by this neural or neurohumoral mechanism.

It is not possible to decide which, if either, of these hypotheses may be correct at present, but it seems unlikely that it would require several hours for thyroxine to diffuse to the anterior pituitary. It seems more reasonable that two separate mechanisms can control thyrotropin secretion, a slower one acting through the central nervous system and a direct one in the anterior pituitary itself.

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RAPID DISAPPEARANCE OF PROGESTERONE FROM OILY SOLUTIONS INJECTED INTRAPERITONEALLY IN MICE[1]

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ABSTRACT

Seventy-five to 22,500 μg. of progesterone dissolved in 1.5 ml. sesame oil were injected intraperitoneally into ovariectomized adult CHI mice. They were exsanguinated two and one-half minutes to eight hours later, and the gestagen levels in the plasma and in the oil recovered from the peritoneal cavities were determined by bio-assay. Concentrations in the plasma were found to have risen quickly and to have declined within an hour. Concentrations in the recovered oil fell to 60 μg. or less within 10 minutes and to about 12 μg. in 30 minutes, regardless of the original concentration of the injected solution.

THIS experiment was undertaken primarily to determine the rate at which progesterone moves out of an oily solution after its intraperitoneal injection. Resulting concentrations of gestagen in the plasma were also investigated. Some of the results have already been briefly reported (1).

PROCEDURE

Young adult CHI mice were bilaterally ovariectomized. Eight days after the operation each mouse received an intraperitoneal injection of sesame oil. Five control mice were injected with 1.5 ml. oil only. Eighty-nine other mice were injected with various quantities of progesterone dissolved in 1.5 ml. oil, as follows: 75 μg., 18 mice; 1,500 μg., 47 mice; 2,250 μg., 6 mice; 7,500 μg., 6 mice; 15,000 μg., 4 mice; 22,500 μg., 8 mice. At a selected interval after the injection each mouse was anesthetized with ether, and the carotid sheath was rapidly exposed and divided. The blood was collected in a 1 ml. centrifuge tube containing 0.05 ml. of 20% sodium citrate solution as an anti-coagulant. The abdomen was opened, and as much injected oil as possible (0.3–0.8 of the original 1.5 ml.) was aspirated. Recovery of the oil sample took an average of about ten minutes. The sample was centrifuged to separate the oil from the small amount (insufficient for assay) of peritoneal fluid also present.

The blood was centrifuged promptly, and a measured volume of plasma was added, drop by drop, with stirring to several volumes of cold (10° C) acetone. The resulting precipitate was separated by centrifugation and was washed three times with equal volumes of acetone and ether. The original supernatant and the washings were pooled, and to them was added a measured volume of sesame oil. The acetone and ether were re-

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moved by distillation. The residue of oil and of water from the plasma was centrifuged to separate the two phases, and the oil was bio-assayed for the acetone- and ether-soluble "free" gestagen according to a standard procedure (2, 3).

The acetone- and ether-insoluble precipitate was re-suspended in a small volume of water, acidified to approximately pH 1, and hydrolyzed for six hours in a boiling water bath to release the "bound" gestagen. The latter was then extracted with ether, taken up in oil, and separately bio-assayed.

The procedure of Hooker and Forbes has been shown to detect not only progesterone but also its biologically active metabolites, Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol, and possibly other, unidentified gestagens as well (4). Gestagen levels may be expressed in Progesterone Equivalents (P.E.). One P.E. is defined as equal to the *activity* of 1 μg. of progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors.

RESULTS

"Free" Gestagen in Plasma. Gestagen was not detected in the plasma of the five mice injected with oil only.

Mice injected with 75 μg. of progesterone were bled at intervals of up to 20 minutes thereafter. Gestagen levels reached 3 P.E./ml. plasma in two mice and were less than 2 P.E./ml. in all others. Plasma concentrations of gestagen declined rapidly after the first five minutes.

Levels of gestagen in the plasma of mice receiving 2,250 μg. or more of progesterone usually fluctuated widely during the half hour in which they were studied. Levels reached a peak (as high as 8.3 P.E./ml. in an exceptional case) in ten minutes and then declined rapidly.

Plasma levels were followed for eight hours in mice receiving 1,500 μg. of progesterone. In the first hour, samples assayed as high as 9.1 P.E./ml. Thereafter, levels also fluctuated widely and erratically, ranging from 6.1 P.E./ml. to less than detectable amounts.

There was no consistent agreement between size of dose and plasma level of gestagen.

"Bound" Gestagen in Plasma. Forty specimens were assayed for "bound" gestagen. Again, plasma levels were not consistent with dose. The highest concentration observed was 4.4 P.E./ml. Usually the levels were much lower, and frequently no "bound" gestagen could be detected.

Gestagen Levels in Recovered Oil. No gestagen was detected in oil recovered from mice injected with oil only.

Results for the other mice are presented in Figures 1 and 2. A striking feature is the remarkable and consistent speed with which the hormone disappeared from the oil. Figure 1 shows that all values for the 75 μg. group and some for the 1,500 μg. group lie close to one curve, while all other values during the first hour lie close to a second curve. Statistical analysis of the values on the second curve showed that the amount of gestagen was reduced by an almost constant percentage for each unit of time. The rate at which gestagen left the oil in mice receiving the lowest doses of hormone, while relatively faster, also appears to be quite consistent. Whether the

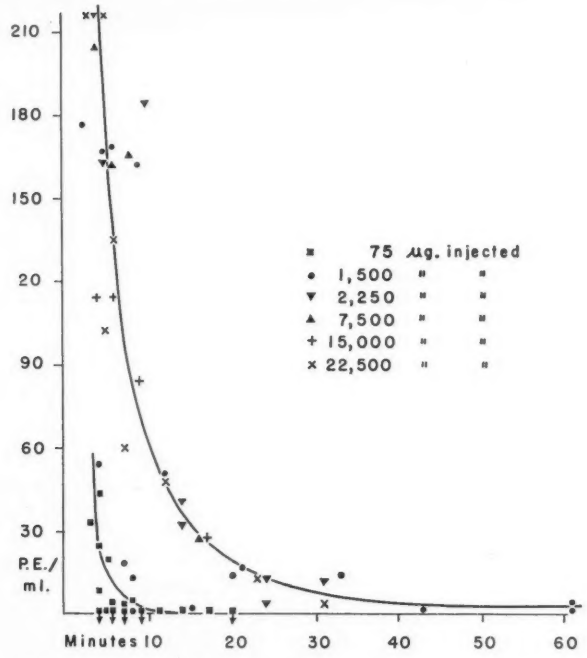

FIG. 1. Graph of assay results for oil recovered during first hour. Each symbol represents the sample from one mouse.

initial dose was 1,000 µg. or 15,000 µg./ml. oil, the concentration (assuming that no progesterone was transformed while in the oil to another compound of different activity) had fallen in ten minutes to approximately 60 µg./ml. oil and in 30 minutes to about 12 µg./ml.

Oil was recovered from mice receiving 1,500 µg. of progesterone not only during the first hour (Fig. 1) but also during the ensuing seven hours (Fig. 2). Thirty mice were killed after the first hour. Oil recovered from 20 of them assayed at low levels lying close to the already established curve. Hormone concentrations in samples from the other 10 mice were considerably higher, levels ranging from 9.5 to 27 P.E./ml. oil; most of the high values were in oil recovered after six hours. However, if the mean value for the second through sixth hour (3.7 P.E.; standard deviation of 2.8 P.E.) and that for the seventh and eighth hours (10.1 P.E.; standard deviation of 12.5 P.E.) are compared, the differences are not statistically significant.

DISCUSSION

The plasma levels of gestagen demonstrate that progesterone or its metabolites reached the blood stream within a few minutes after the former substance was injected intraperitoneally. Nevertheless, mere escape of

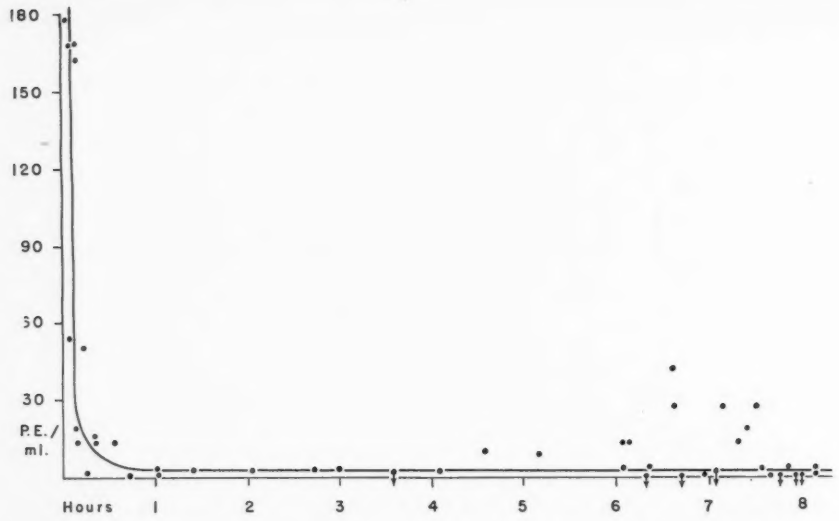

FIG. 2. Graph of assay results for oil recovered during eight hours in mice injected with 1,500 μg. of progesterone. Each symbol represents the sample from one mouse.

the progesterone into the plasma cannot in itself account for the hormone's swift disappearance from the oil. The total plasma volume of the young adult mouse is only perhaps 1 ml., and can contain at most only a few micrograms of gestagen.

Hepatic inactivation of progesterone occurs in the mouse (5), and undoubtedly accounted for the disappearance of some of the injected hormone. What happened to the remainder of the progesterone is not clear. The total area of the parietal and visceral peritoneum of the mouse is appreciable. It may be that much of the hormone adhered to or was absorbed by the peritoneal cells. It is possible that a part of the progesterone was taken up in the fat; concentrations of up to 2.3 and 4.4. μg. per gram of fatty tissue have been found in rabbits and women, respectively (6, 7). The fact that in some mice the concentration of the oil solution apparently rose during the seventh and eighth hours suggests partial return of the hormone to the oil. It is also possible that the administered progesterone, instead of leaving the oil, was transformed in the oil into a compound not detected by the bio-assay, but such transformation in this site appears unlikely.

The remarkable speed and seemingly steady rate at which the hormone escaped from the oil are interesting. Plotz and Davis (8) found that C^{14}-4-progesterone apparently moves rather quickly out of its oily solution after injection of the latter into human beings. In mice it appears that intraperitoneally injected concentrations of 50 to 1,000 μg./ml. oil are disposed of at one rate, and concentrations of 1,000 to 15,000 μg./ml. at another.

Data for the first curve were too scanty for statistical treatment. The second curve reveals a fairly uniform per cent loss per minute, implying that a mechanism relatively unaffected by biological variation may have been responsible for movement of the hormone out of the oil.

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A COMPARISON OF THE INHIBITORY EFFECTS OF 2-METHYL-1,2-BIS(3-PYRIDYL)-1-PROPANONE AND AMPHENONE B ON ADRENAL CORTICAL SECRETION IN THE DOG[1]

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ABSTRACT

In short term experiments in the dog 2-methyl-1,2-bis(3-pyridyl)-1-propanone (SU 4885) in high dosage totally suppressed the secretion of steroids by the adrenal gland. In low dosage there was a shift from cortisol and corticosterone to 11-desoxycortisol (Reichstein's Compound S) and desoxycorticosterone suggesting a selective inhibition of hydroxylation at C_{11}. In contrast amphenone suppressed total steroid secretion (*i.e.*, of cortisol and corticosterone) with no evidence for a selective action on 11-hydroxylation.

THE demonstration by Nelson and Woodard (1) that the administration of the insecticide DDD (2,2-bis (parachlorophenyl), 1,1-dichloroethane (Fig. 1) caused adrenal cortical atrophy in the dog, provided an indication that, at least in this species, the adrenal gland may be inhibited by a synthetic non-steroidal substance, although recent work by Cueto *et al.* (2) suggests that the ortho-para analogue was the active principle of impure DDD used in earlier experiments (1). Other related compounds such as Perthane (Fig. 1) have been studied by Taliaferro and Leane (3) and Larson *et al.* (4) who found that relatively minor changes in the chemical structure altered the action on the adrenal cortex.

The adrenal inhibitor which has been most extensively investigated is amphenone B (3,3-bis (p-aminophenyl)-2-butanone). Studies by Hertz (5) and other workers have shown that this substance inhibits adrenal steroid secretion both in the dog and in man, although the histological appearance of the adrenal cortex after the administration of the drug was not atrophy but hyperplasia. Recently, the compound 2-methyl-1, 2 bis (3-pyridyl)-1-propanone (SU 4885 or DPP) has been synthesized by Allen

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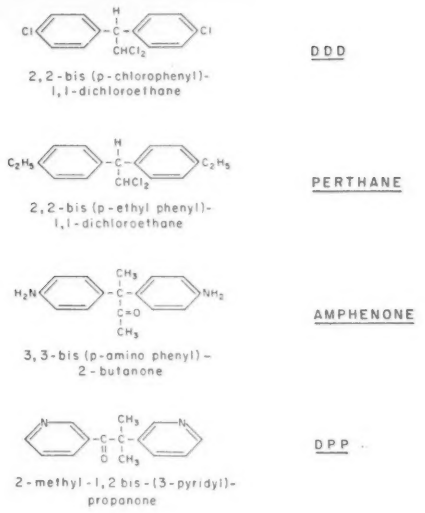

FIG. 1. Pharmacologic inhibitors of adrenal function (DPP=SU 4885).

and Bencze (Fig. 1) and preliminary results obtained by Chart *et al.* (6) indicated that this drug was effective as an inhibitor of adrenal steroid secretion in experimental animals. The ability of this substance to selectively inhibit oxygenation at the 11-position of the steroid molecule has been described (7, 8). The present report is concerned with an investigation of the effects of SU 4885 on adrenal cortical function in the dog and a comparison of these effects with those produced by amphenone.

METHODS

Mongrel dogs weighing 20–25 kg. were used. The right lumbo-adrenal vein was cannulated under sodium pentobarbital anesthesia according to the method of Nelson and Hume (9) and the adrenal venous blood was collected at timed intervals. An infusion of 25 units of hog ACTH in 500 ml. of normal saline was administered through the femoral vein, to ensure constant maximal adrenal cortical stimulation. Total 17-hydroxycorticoids in the blood were estimated by the method of Nelson and Samuels (10).

Characterization of individual steroids was carried out by extracting the plasma twice with equal volumes of ethyl acetate, washing first with 1N sodium hydroxide, then with water, drying with anhydrous sodium sulfate and evaporating to dryness. As much lipid material as possible was removed by partitioning the extract between hexane and 70% aqueous methanol. The methanol layer was removed, evaporated to dryness, redissolved in a methanol-chloroform 1:1 solution and applied to a paper chromatogram. The chromatograms were run in the "B3" and "B5" systems of Bush (11), and the toluene-propylene-glycol system of Zaffaroni (12). Steroids were identified by means of the running rates of the free and acetylated substances compared with known standards, ultraviolet light absorption, the soda fluorescence test (11), reactions with blue tetrazolium and phenylhydrazine reagents, and absorption spectra in concentrated sulphuric acid. Quantitation was carried out by eluting the steroids from the paper with methanol, and using the phenylhydrazine reaction of Porter and Silber (13) for the hydroxycorticoids and the blue tetrazolium method of Mader and Buck (14) for the other steroids.

RESULTS

When a single injection of 75 mg./kg. of SU 4885 bitartrate was given intravenously the total 17-hydroxycorticoids in the adrenal-venous blood fell to approximately 10% of the initial value within 10 minutes, rising to 75% within 3 hours as is shown in Figure 2. The effect of a small dose of the drug, 5 mg./kg. given as an infusion over 30 minutes is seen in Figure 3. In this case no decrease in 17-hydroxycorticoids occurred and in fact a suggestive rise was observed. When the infusion was increased to 75 mg./kg. a reduction in steroid output was again obtained.

The effect of a single intravenous injection of amphenone hydrochloride, in a molar dosage equivalent to 75 mg./kg. of SU 4885 bitartrate, is illustrated in Figure 4. The observed decrease in 17-hydroxycorticoids and the subsequent return toward the initial values was quantitatively similar to that obtained with SU 4885. It was noted, however, that during the course of the experiments, the anesthetic effect of SU 4885 was less than that of amphenone, since more pentobarbital was necessary to maintain the required state of anesthesia. Differences in the action of the two drugs became most apparent when the individual steroids were studied. A single injection of 75 mg./kg. of SU 4885 was given and the average of results from three experiments is seen in Figure 5. Cortisol fell from 7.0 μg. per minute to immeasurable levels within 10 minutes but even after 7 hours, the value was only 0.9 μg. per minute, returning to the initial level by 20

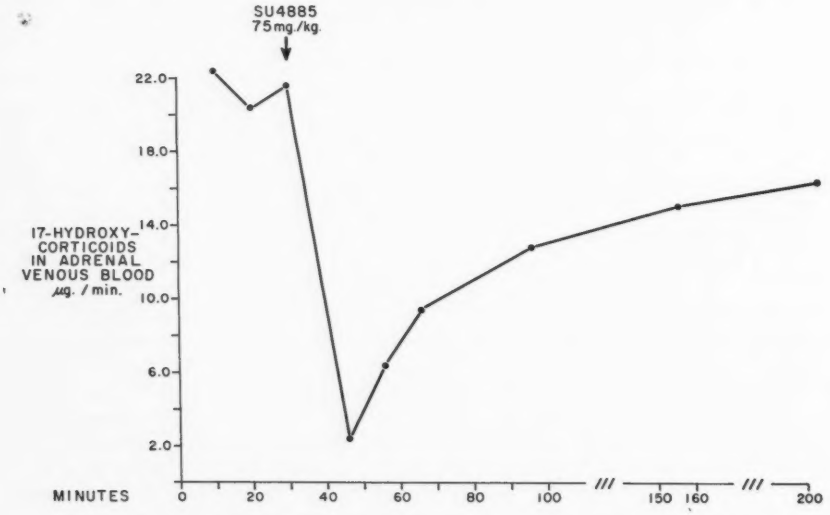

FIG. 2. The effect of a single intravenous injection of SU 4885 on total 17-hydroxycorticoid secretion in the adrenal vein of the dog.

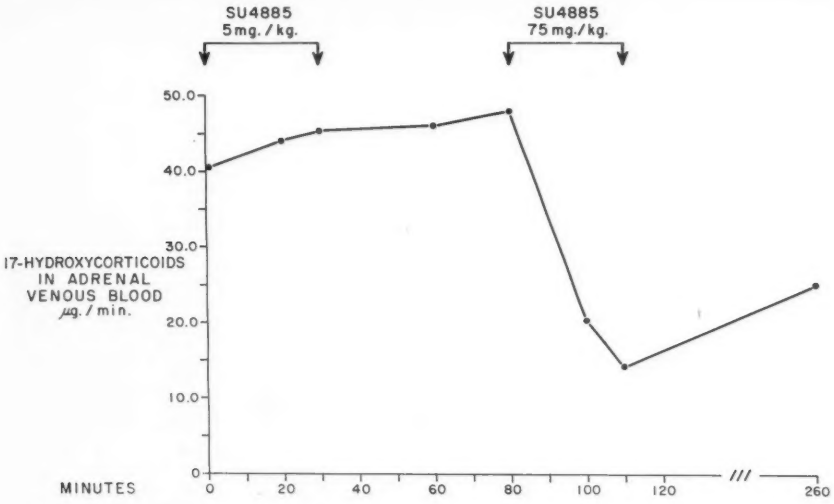

FIG. 3. The effect on total 17-hydroxycorticoid secretion in the dog of 30 minute infusions of SU 4885 in doses of 5 mg./kg. and 75 mg./kg.

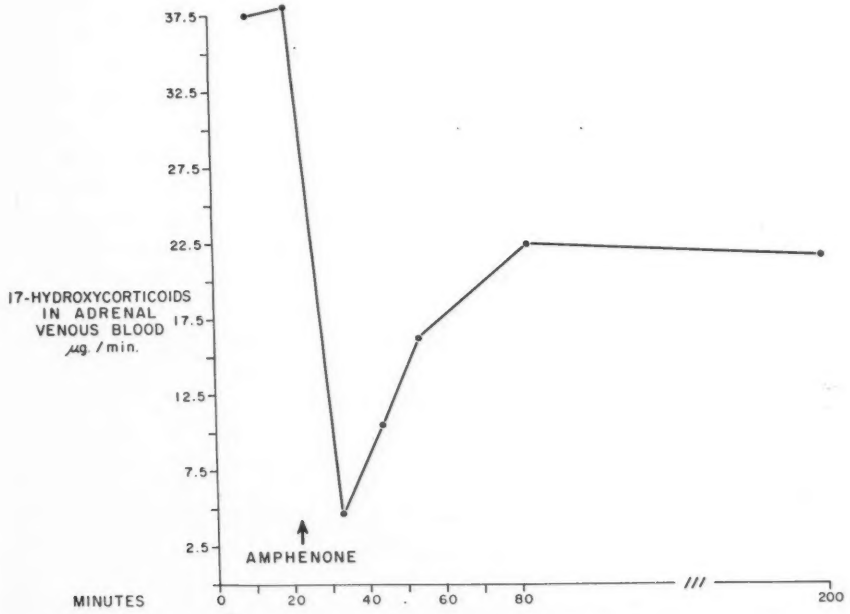

FIG. 4. The effect of a single injection of amphenone on total 17-hydroxycorticoids secreted into the adrenal vein of the dog.

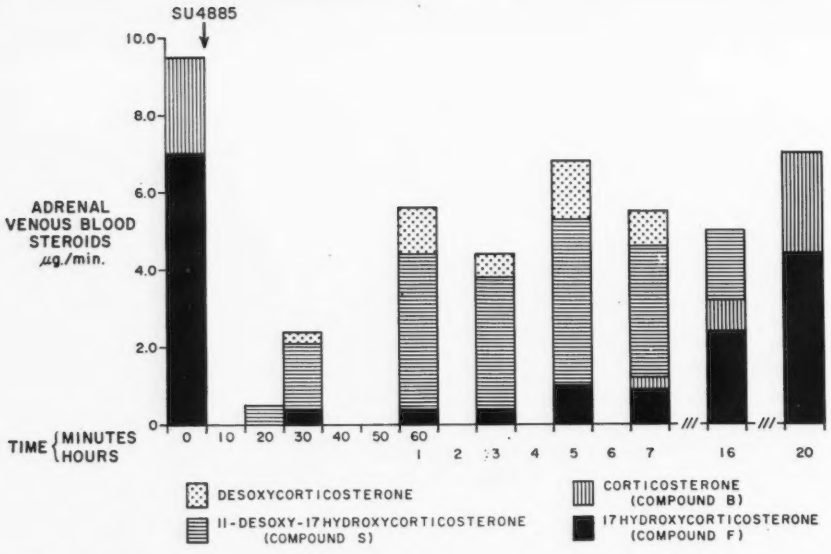

FIG. 5. Mean values for secretion of 17-hydroxycorticosterone (solid bars), corticosterone (vertical crosshatch), 11-desoxy-17-hydroxycorticosterone (horizontal crosshatch), and desoxycorticosterone (stippled) in three dogs given 75 mg./kg. SU 4885 intravenously in a single injection.

hours. Equally striking was the appearance of 17-hydroxy, 11-desoxy corticosterone (Reichstein's Compound S) which rose from previously undetectable levels to about 4 μg./min., 1–5 hours after the injection of the drug. It would appear that levels of Compound S are maximal by one hour and are maintained to at least 7 hours. Corticosterone, which was initially present in approximately half the quantity of cortisol disappeared and seemed to be replaced by 11-desoxycorticosterone.

In contrast with these results, Figure 6 shows the effect of amphenone in a dosage equivalent to 75 mg./kg. of SU 4885. A temporary reduction in cortisol and corticosterone was observed and during the following three hours the levels of both steroids gradually rose but without the appearance of 11-desoxy-compounds.

DISCUSSION

These findings indicate that SU 4885 has an inhibitory effect on adrenal cortical secretion in the dog. This action is not mediated by an interference with endogenous ACTH secretion since ACTH was administered during the course of each experiment. Quantitatively the effect on total 17-hydroxycorticoids seemed to be similar to that of amphenone, when given in an equivalent dosage, although the anesthetic effect of SU 4885 was

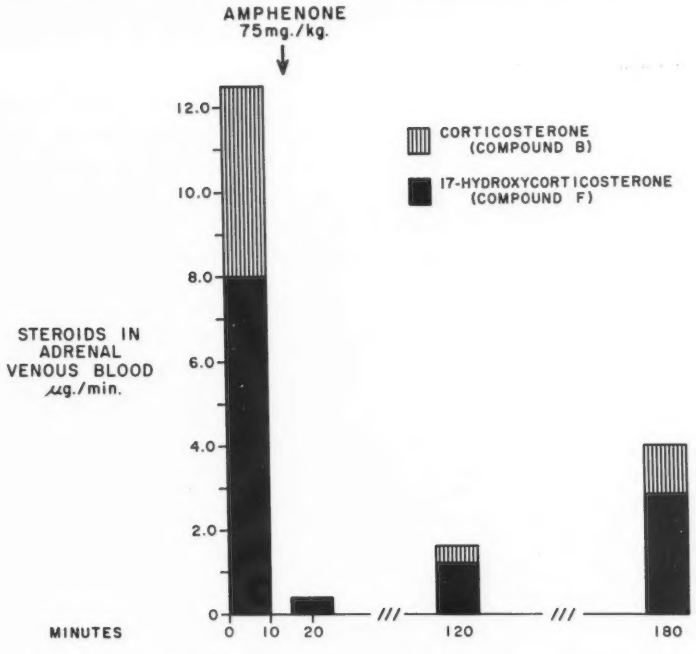

FIG. 6. The effect of a single injection of amphenone (75 mg./kg.) on secretion of corticosteroids in the dog.

less marked. The most important differences, however, were seen in the action of the two drugs on the individual steroids. In large doses SU 4885 caused a fall in total steroid secretion but this was followed by a prolonged inhibition of cortisol and corticosterone, lasting at least 7 hours, with the appearance of increasing amounts of Compound S and desoxycorticosterone. In smaller doses, no reduction in total 17-hydroxy steroids occurred but as was previously reported inhibition of cortisol occurred, with the concomitant secretion of large quantities of 11-desoxycortisol, indicating that the adrenal 11-hydroxylation enzyme system was especially sensitive to the effects of this drug (7, 8). The action of SU 4885 is in contrast with that of other known adrenal inhibitors. Studies with DDD (2) showed that reduction in 17-hydroxycorticoids in the dog following the administration of this drug was accompanied by actual destruction of adrenal cortical tissue. Amphenone (4) and SU 4885 (6) caused an increase in adrenal size. The experiments described above, demonstrate that amphenone produced a temporary suppression of cortisol and corticosterone but without evidence of any selective action on 11-hydroxylation. This is in agreement with the findings of Rosenfeld and Bascom (15), who showed, as a result of

adrenal perfusion experiments, that amphenone caused a generalized inhibition of adrenal steroid secretion by interference with a number of different enzymes.

The selective action of SU 4885 is, therefore, of considerable interest, although the exact mode of its activity remains to be elucidated. Tomkins *et al.* (16) have presented evidence that the 11β-hydroxylase system is complex, at least three enzyme factors being involved, so that the drug could have one or several sites of action. Further study of the effects of this compound is indicated and may lead to increased knowledge of the mechanism of adrenal steroid biosynthesis.

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DEMONSTRATION OF THE CONCENTRATION OF ASTATINE-211 IN THE MAMMARY TISSUE OF THE RAT[1]

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ABSTRACT

Concentration of At^{211} in the mammary tissue of virgin and lactating female rats has been demonstrated both by gross radioactive assay and by autoradiography. Secretion of At^{211} in the milk was shown by its presence in the gastrointestinal tracts and carcasses of nurslings and by the presence of alpha-particle tracks in glandular lumens in autoradiographs. In virgin mammary tissue At^{211} was chiefly associated with the parenchyma, although small amounts could also be found in the stroma.

INTRODUCTION

WHEN 55-day old female Sprague-Dawley rats were given 0.5 μc./gm. body weight of At^{211}[2](1), 75% of the At-injected rats developed mammary tumors primarily of duct origin during the ensuing year. Only 17% of non-injected control rats developed mammary tumors during this same interval (2–3). In the elucidation of the cause of this augmented tumor incidence, it was of paramount importance to investigate the possible role of direct alpha-particle irradiation of the immature mammary tissue. There were indications from earlier work that At^{211} might concentrate in mammary tissue (4). The metabolism of At^{211} resembles that of I^{131} in many respects, and gross assays had shown that both halogens tended to concentrate in the skin and subcutaneous tissues (4). Further, Rugh (5) and Brown-Grant (6) have demonstrated the capacity of the mammary gland to concentrate I^{131} and to secrete it in milk.

Anatomically, the breast tissue of the virgin rat consists of a simple duct system terminating in tiny alveolar buds, embedded in, and surrounded by a copious amount of connective tissue and fat (7). Gross dissection of vir-

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[1] This work was performed under the auspices of the U. S. Atomic Energy Commission.

[2] At^{211} is an isotope of the heaviest halogen with a half-life of 7.5 hours. It decays by emission of alpha particles with a mean energy of 6.3 Mev and by electron capture (1). In the latter process 80 Kev x-rays are emitted allowing the detection of this isotope with photosensitive scintillation equipment.

ginal mammary parenchyma is virtually impossible; thus radioactive assay was inconclusive for assessing the concentration of At^{211} in mammary tissue. The At^{211} found in the mammary region might merely be associated with blood vessels, lymphatic tissue, or fat. Autoradiographs, however, provide a means by which the presence of At^{211} in the mammary parenchyma can be demonstrated visually. Because of the possibility of movement of At^{211} from its site of deposition in the living tissue during the processes of fixation and dehydration, it was also necessary to study the distribution of At^{211} in the lactating mammary gland and to determine whether At^{211}, like I^{131}, is secreted in the milk.

METHODS

At^{211} was prepared by the method of Parrott *et al.* (8). Three 43-day old virgin female Sprague-Dawley rats and two lactating Long-Evans rats each received 200 μc. of At^{211} intraperitoneally. After injection, the lactating rats were returned to their litters. Four hours later all the At-injected rats and the nurslings of the injected mothers were sacrificed with chloroform. This experiment was repeated with identical groups of virgin and lactating rats, except that the animals were sacrificed three hours after the At^{211} injection. Samples of mammary tissue were dissected from both inguinal regions of each of the injected rats, and were fixed in Bouin's fluid. One portion was dehydrated with dioxane, embedded in paraffin, sectioned at 6μ, and mounted on Eastman's 10μ NTA stripping film for autoradiographs. The remaining portions of the mammary tissue were weighed and assayed for At^{211} x-ray activity with a NaI-Tl$\downarrow$ crystal scintillation counter.

The gastrointestinal tracts and thyroids of 4 nurslings from the litters in Experiment 1 were dissected and assayed, and when present, bladder urine was also assayed. To exclude the possibility that any radioactivity present was due to external contamination, the remaining nurslings were skinned, and carcasses were assayed separately. In the second experiment all the nurslings were skinned and the gastrointestinal tracts dissected. These samples, and the residual carcasses were assayed separately.

RESULTS

The measurements of At^{211} radioactivity in mammary tissue of virgin and lactating rats are shown in Table 1. The 1-hour difference in post-injection interval did not affect the At^{211} concentration, percent of injected dose per gram of wet tissue (%/gm.), in the virginal tissue. The mean for all animals was 0.38%/gm. On the other hand, the At^{211} concentration in the lactating mammary tissue was appreciably higher at the earlier interval. This difference is reflected in the amount of At^{211} transferred to the nurslings at the 2 intervals (See Table 2). The total passed to the litters was twice as much 4 hours after injection as after 3 hours. At the 3-hour interval the gastrointestinal tracts contained 35% of the At^{211} found in the young. One hour later, while the total At^{211} in the nurslings had doubled, the gastrointestinal concentration was reduced to 25%, indicating rapid absorption of At^{211} from the infant gastrointestinal tract. The relatively low skin concentrations indicated that contamination from such sources

TABLE 1. DEPOSITION OF At^{211} IN TISSUE FROM THE MAMMARY LINE OF IMMATURE VIRGIN FEMALE RATS AND LACTATING RATS 3 TO 4 HOURS AFTER INTRAPERITONEAL INJECTION

Animal	Postinjection interval	Sample wt., Gm.	% injected At^{211}	At^{211} concentration %/gm.
Virgin rats				
a	3 hours	.25	.08	.31
b		.25	.14	.57
c		.68	.27	.40
d	4 hours	.35	.13	.36
e		.33	.10	.29
f		.49	.17	.34
Mean				.38
Lactating rats				
a	3 hours	1.69	1.47	.87
b		1.34	1.01	.75
c	4 hours	.95	.48	.50
d		.82	.32	.40

as the mother's urine was negligible, and that nearly all of the At^{211} found in the young had been transferred in the milk.

The distribution of At^{211} in the mammary glands was studied autoradiographically. In the glands from the virginal rats the tissue was preponderantly adipose. Scattered about within this stroma were small ducts and alveolar buds of epithelial structure. Small aggregates of lymphoid tissue were sometimes seen. The autoradiographs showed that the alpha-particle tracks most commonly arose from the epithelium, whether ductal

TABLE 2. MATERNAL TRANSFER OF At^{211} BY WAY OF THE MILK

Litter	Experiment 2 (3 hours)		Experiment 1 (4 hours)	
	No. of specimens	% of Mother's At^{211} dose	No. of specimens	% of mother's At^{211} dose
	a.		c.	
Whole nurslings	6	1.21	2	2.63
Skin only	6	.12	2	.32
GI Tract	6	.78	4	.90
Thyroid			4	$<.01$
Bladder urine			4	.02
Total passed to litter	(6)	7.3		15.8
	b.		d.	
Whole nurslings	6	.58	8	.84
Skin only	6	.07	8	.11
GI Tract	6	.33	4	.35
Thyroid			4	$<.01$
Bladder urine			4	$<.01$
Total passed to litter	(6)	3.5	(12)	10.1

(Fig. 1) or alveolar (Fig. 2). Tracks were very rare in the lumens of these structures. The adipose tissue also showed some At^{211}, but tracks were only about one-fifth as frequent. They arose from within the fat-containing space of the adipose cells. Tracks were also seen arising directly from the fibroblasts of the stroma. Blood vessels were scanty in this non-secreting gland, but tracks could sometimes be seen arising in the lumen of small vessels. The lymphatic tissue showed no tracks.

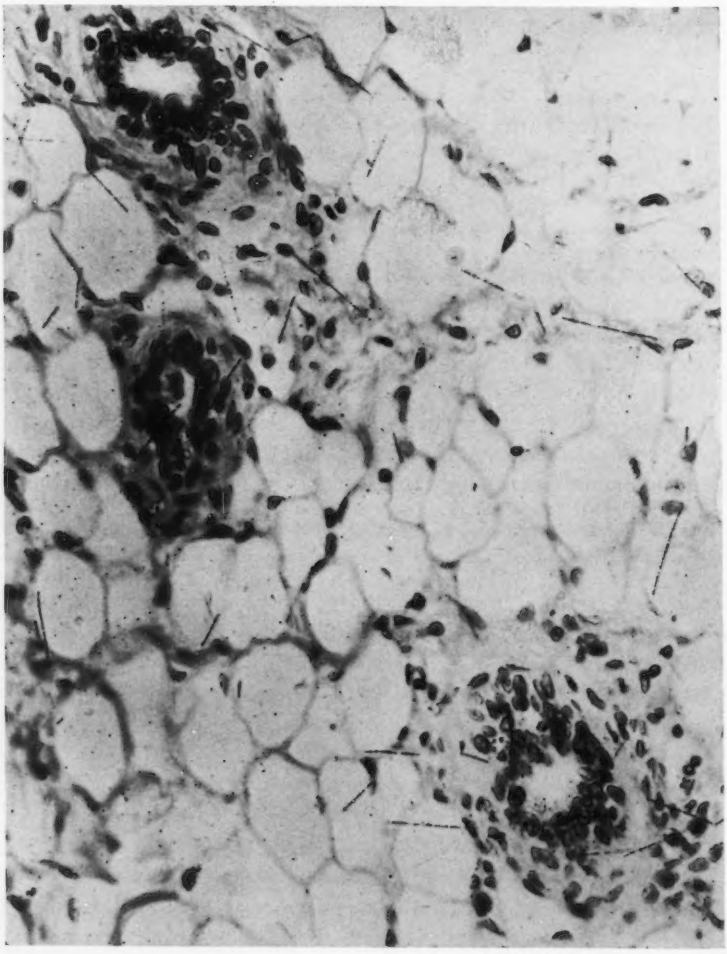

FIG. 1. Stripping film autoradiograph (NTA) of At^{211} localization in mammary tissue of 55-day old virginal rat. H and E counterstain; magnification 300×. Concentration in ducts and periductal tissue; sparse tracks in adipose connective tissue.

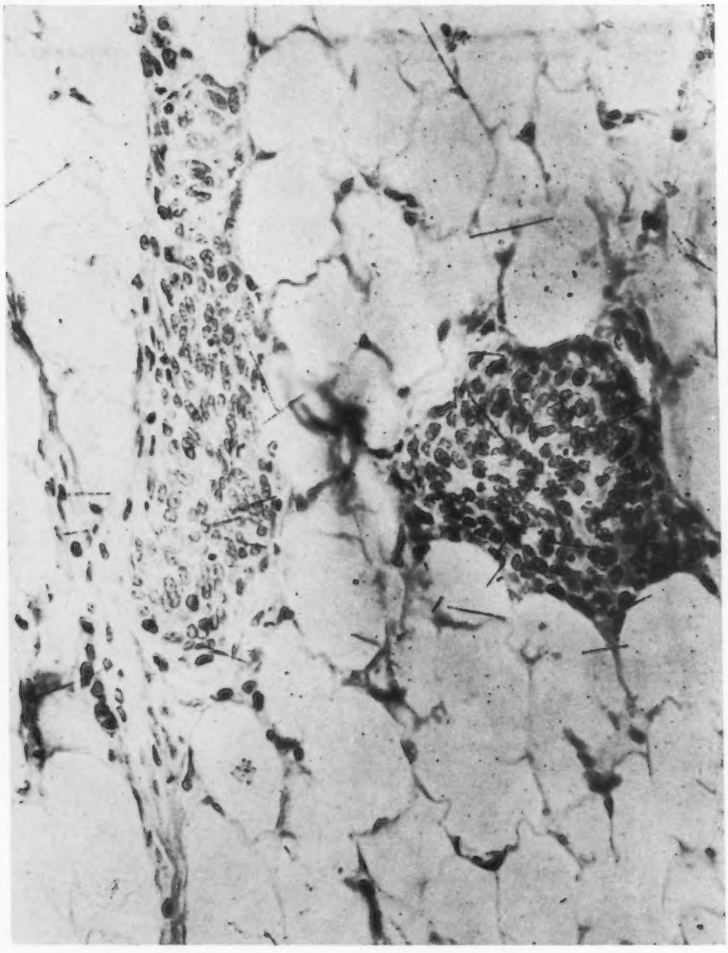

FIG. 2. Association of At^{211} with acinar tissue of mammary gland of 55-day old virginal rat. Technical data as in Figure 1.

In a typical autoradiograph from a lactating rat (Fig. 3) the tissue was predominantly glandular; the stroma contained an abundant vasculature and scanty adipose tissue. The acini of the glands were in varying stages of secretion, some being composed of heightened epithelium and a relatively small lumen, while in others the cells were lower, and the acini were dilated with secretion. In some acini tall cells were seen whose distal portion (i.e., toward the lumen) was distintegrating in the typical fashion of apocrine secretion. Lymphatic tissue could also be seen in these glands.

FIG. 3. At^{211} localization in mammary tissue of lactating rat. Note tracks in stroma, epithelium and lumens. Technical data as in Figure 1.

The autoradiographs showed tracks originating in the acinar epithelium. In the cells showing disintegration of the distal portion, tracks were often found originating in this part of the cell. Correspondingly, some tracks were seen in the lumen when it contained secretion. Tracks could also be seen arising from cells which were not yet actually releasing secretion. The blood vessels often showed a few tracks originating in the lumen and occasionally from the tissue of the walls. Tracks were rarely found in fat cells. The lack of tracks arising from lymphatic tissue of either group pro-

vided evidence that no appreciable translocation of the At^{211} had occurred during the technical manipulations.

DISCUSSION

The localization of At^{211} in lactating glandular tissue and its transfer to nursing young via the milk parallels demonstrations of radioiodine in milk. It is, of course, well established that casein may be iodinated *in vitro*, and it is possible that At^{211} is bound in comparable fashion. However, the possibility that it is associated with the fat of milk should not be neglected.

The epithelial localization in the non-lactating, virginal gland is of substantial interest in view of the frequency of occurrence of neoplasms of duct origin when such animals receive At^{211}. Of no less interest is the occasional uptake by fibroblasts in such animals, for fibromata and sarcomata are not infrequently seen, and tumors showing both epithelial and connective tissue hyperplasia are very common.

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THE EFFECT OF ADRENAL STEROIDS ON PERIPHERAL FAT MOBILIZATION IN THE RAT[1]

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ABSTRACT

The fasting adrenalectomized rat moved more fat out of the epididymal fat depot during a 12-hour fast than did the normal animal. Restoration of cortical hormone (hydrocortisone) at both high and low dose levels elicited a marked inhibition of fat movement out of this depot. The administration of hydrocortisone to intact animals also produced this inhibition. The procedure of sham adrenalectomy caused a significant diminution of fat mobilization from the epididymal depot for at least six days following the laparotomy. This response to trauma has now been observed in another laboratory following a burn stress. There are probably factors other than the release of cortical steroids which mediate this response but an increase in steroid titer appears to alter fat mobilization in this direction. The hypophysectomized animal behaves differently from the adrenalectomized animal in this regard. The amount of fat moved out of the epididymal depot by the hypophysectomized animal during a 12-hour fast was much smaller than that moved by either the intact or adrenalectomized animal. Administration of cortical hormones alone did not restore the rate of fat movement to normal. Work is in progress using combinations of target gland hormones and growth hormone in hypophysectomized animals.

ADRENALECTOMY inhibits the protein mobilization of stress (1) and the accumulation of fat by a damaged liver (2, 3). It has been suggested therefore, that adrenal insufficiency is accompanied by a simultaneous inhibition of fat mobilization out of peripheral fat depots. There are data which do not support this suggestion as regards depot fat movement during a fasting period (4, 5). Adrenalectomized rats appear to lose more rather than less fat from certain fat depots than do intact animals fasted for varying periods of time (6 to 48 hours) (5). We have investigated the effects of hydrocortisone on the total fat changes in the epididymal fat depot of the rat during fasting and feeding. Our data indicate that a plethora of cortical steroids inhibits fat movement out of this depot even during a fast in intact and adrenalectomized animals. Further, a deficiency of steroids augments fat movement.

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METHODS

Male Sprague-Dawley rats weighing 90–110 gm. were used throughout. Larger animals do not show comparable symmetry of the epididymal fat depots (5). In all experiments, each animal was used as its own control. The first epididymal fat body was removed immediately before the onset of the fasting period and the second at the end of the fast. Total lipids in each depot were determined by the method of Bloor (6). The difference between the amount of extractable lipid in the depots, before and after the fast, per 100 mg. of initial depot weight, was taken to indicate the degree of fat mobilization. The epididymal fat bodies were removed under ether anesthesia by orchiectomy through a lateral scrotal incision (5). All animals were maintained on Purina rat chow and water *ad libitum* with the exception of the adrenalectomized animals that were given 0.9% saline in place of tap water. Data are reported from only those adrenalectomized and hypophysectomized animals that did not show any adrenal or pituitary tissue on post mortem examination.

Intact, sham adrenalectomized, adrenalectomized (6 days postoperative) and hypophysectomized (11 and 217 days postoperative) rats were fasted for 12 hours and the total lipid content of the epididymal depots was determined.

Intact, sham adrenalectomized, and adrenalectomized rats were given either 0.3 mg. hydrocortisone or 1.0 mg. hydrocortisone subcutaneously for three successive days just prior to the start of a 12-hour fast. Hypophysectomized animals were given 0.3 mg. hydrocortisone one hour prior to the fasting period. Quantitative fat changes in the epididymal depot were determined.

RESULTS

Initial weight of the epididymal fat depot during ad libitum feeding: All animals were in the same weight range (90–110 gm.) when the first depot was removed. Despite this similarity in total body weight, there were significant differences in the amounts of fat deposited on the ad libitum feeding regime. In a total of 157 intact, untreated rats, the amount of extractable lipid from a single depot was 83.8 ± 1.5 mg. (S.E.). For 96 adrenalectomized animals the extractable lipid was 46.9 ± 1.7 mg. and for 59 sham adrenalectomized animals it was 69.2 ± 2.3 mg. Eleven days after hypophysectomy, the extractable lipid (8 animals) was 110.4 ± 7.0 mg. and in the series maintained for 217 days after hypophysectomy (8 animals) the lipid content was 187.9 ± 8.5 mg. It is apparent that adrenalectomized animals fed ad libitum do not deposit as much fat in this depot as do intact animals, while hypophysectomized animals deposit increasingly greater amounts than normal (Table 1).

The administration of hydrocortisone to intact, sham adrenalectomized and adrenalectomized animals for 3 days (during which time the animals were allowed free access to food and fluids) produced significant changes in the amount of fat deposited in the epididymal depots. At the two dose levels of hydrocortisone used in these experiments all the animals showed increased fat accumulation in the depot. The total body weight, however, did not change in the same direction. The animals did not show a significant weight gain and at the higher dose level of hydrocortisone (1.0 mg./day) many animals lost weight. In spite of this, the extractable lipids in a single depot in intact animals given 0.3 mg. hydrocortisone rose to $97.6 \pm$

TABLE 1. INITIAL WEIGHT OF EPIDIDYMAL FAT BODIES
(After *ad libidum* feeding)

Experimental procedure	No. of animals	Mg. of extractable fat	S.E.
Intact	157	83.8	1.5
Sham Adrenalectomy	59	69.2	2.3
Adrenalectomy	96	46.9	1.7
Hypophysectomy—11 days after operation	8	110.4	7.0
Hypophysectomy—217 days after operation	8	187.9	8.5
Intact—0.3 mg. hydrocortisone/day for 3 days	12	97.6	6.1
Intact—1.0 mg. hydrocortisone/day for 3 days	12	119.4	7.1
Sham Adrenalectomy—0.3 mg. hydrocortisone/day for 3 days	21	80.8	3.8
Sham Adrenalectomy—1.0 mg. hydrocortisone/day for 3 days	33	109.4	2.8
Adrenalectomy—0.3 mg. hydrocortisone/day for 3 days	19	75.8	3.5
Adrenalectomy—1.0 mg. hydrocortisone/day for 3 days	27	83.3	4.4

S.E. = Standard Error.

6.1 mg. With 1.0 mg. hydrocortisone, fat levels rose to 119.4 ± 7.1 mg. as compared to the untreated animals' 83.8 ± 1.5 mg. A similar increase was observed in the sham adrenalectomized animals. Thus, with 0.3 mg. hydrocortisone, the amount of extractable lipid was 80.8 ± 3.8 mg. With 1.0 mg. hydrocortisone, the lipid content was 109.4 ± 2.8 mg. as compared to the untreated sham adrenalectomized animals' value of 69.2 ± 2.3 mg. The adrenalectomized animals responded to hydrocortisone in the same fashion. The untreated animals had 46.9 ± 1.7 mg. of extractable fat in the depot. After treatment with 0.3 mg. hydrocortisone the amount of fat was found to be 75.8 ± 3.5 mg. and after a regime of 1.0 mg. of hydrocortisone, was 83.3 ± 4.4 mg. per depot (Table 1).

Changes in lipid content resulting from a 12 hour fast: It was found that intact animals fasted for 12 hours mobilized 13.0 ± 2.4 mg. fat/100 mg. initial depot fat. Adrenalectomized animals mobilized significantly more fat during this period: 26.3 ± 2.2 mg. fat/100 mg. initial depot fat. Hypophysectomized animals showed no significant mobilization of fat during this brief fasting period (Table 2). It is of interest that in these experiments the sham adrenalectomized animals also showed little or no quantitative fat changes in response to the withdrawal of food. A possible explanation for this will be discussed.

It can be seen from Table 2 that the administration of hydrocortisone to both intact and adrenalectomized animals effectively prevented the rapid mobilization of fat out of the depots that was observed in the untreated animals. It is difficult to evaluate the data obtained from the sham adrenalectomized animals because the procedure of sham adrenalectomy alone appears to inhibit fat mobilization within this time interval. The administration of hydrocortisone to hypophysectomized animals resulted

TABLE 2. MOBILIZATION OF EPIDIDYMAL FAT DURING A FASTING PERIOD

Experimental Procedure (12-Hour Fast)	No. of rats	Initial depot weight, mg.	Depot weight after, mg.	Mobilization mg.	S.E.	t	P	Mobilization, mg./100 mg. initial depot wt.	S.E.	t	P
Intact—saline	17	90.4	79.0	+11.4	2.4	8.1	<0.001	+13.0	2.4	9.5	<0.001
Intact—0.3 mg. hydrocortisone	12	97.6	115.1	−17.5	2.5			−18.2	2.5		
Intact—saline	17	90.4	79.0	+11.4	2.4	4.4	<0.01	+13.0	2.4	5.3	<0.01
Intact—1.0 mg. hydrocortisone	12	119.4	124.7	− 5.3	3.1			− 6.1	3.1		
Sham adrenalectomy—saline	16	70.8	69.9	+ 0.9	1.3	1.0	Not sig.	+ 2.5	2.8	1.3	Not sig.
Sham adrenalectomy—0.3 mg. hydrocortisone	11	80.8	76.8	+ 4.0	0.9			− 2.3	2.1		
Sham adrenalectomy—saline	16	70.8	69.9	+ 0.9	1.3	0.5	Not sig.	+ 2.5	2.8	1.4	Not sig.
Sham adrenalectomy—1.0 mg. hydrocortisone	20	109.4	106.5	− 2.9	0.8			− 3.3	3.0		
Adrenalectomy—saline	30	39.5	29.3	−10.2	1.4	5.0	<0.001	+26.3	2.2	7.0	<0.001
Adrenalectomy—0.3 mg. hydrocortisone	9	76.5	79.6	− 3.1	3.6			− 4.9	4.6		
Adrenalectomy—saline	30	39.5	29.3	+10.2	1.4	6.5	<0.001	+26.3	2.2	7.4	<0.001
Adrenalectomy—1.0 mg. hydrocortisone	15	75.0	76.0	− 1.0	1.8			− 2.7	3.0		
Hypophysectomy—11 days	8	110.4	105.8	+ 4.6	3.0			+ 3.5	6.3		
Hypophysectomy—217 days	8	187.9	191.8	− 3.9	5.6			− 2.0	5.6		

+ = Mobilization.
− = Deposition.
S.E. = Standard Error.

in ambiguous data and these experiments are being repeated with chronic rather than acute pretreatment with hydrocortisone.

DISCUSSION

Despite the accumulation of data concerning the metabolic effects of the cortical steroids, there remains uncertainty as to the mechanism of action of these hormones. For example, the regulation of fat metabolism during a fasting period has been attributed, in part, to the mobilizing action of the cortical steroids on the fat depots. This point of view is supported by data on protein mobilization (1) which suggests that the adrenalectomized animal cannot move protein from peripheral tissues to the liver when a caloric demand is imposed.

The fasting adrenalectomized animal is thus a more precarious animal in terms of available caloric substrate for metabolic needs. The hypoglycemia observed in such animals reflects this deficit. It has been suggested that this deficit is aggravated further by a concommitant defect in the availability of fat moieties from peripheral fat depots (7). The evidence for this is based largely on data obtained by measuring the degree of fat accumulation in the liver. It is clear that the adrenalectomized animal does not develop a fatty liver under circumstances which produce this effect in intact animals (2, 3). The level of liver lipid, however, is a resultant of many factors and does not always reflect the degree of fat mobilization from the depots. Thus, Gillman and Gilbert have found that

in the baboon, the depletion of fat depots after adrenalectomy was not accompanied by the accumulation of fat in the liver (8). Earlier work from our laboratory also indicates that adrenal insufficiency produces a relative deficiency in protein mobilization while at the same time it increases the rate of fat mobilization (5). The level of liver fat does not mirror the peripheral fat depot behavior in this case.

In the experiments reported here, it was found that the adrenalectomized animal differs markedly from the hypophysectomized animal with regard to peripheral depot fat responses to feeding. Both types of preparations are known to resist fatty infiltration of the liver. In the case of the adrenalectomized animal, this phenomenon exists together with an augmented movement of fat out of the depot during fasting and a decreased fat accumulation in the depot during feeding, as compared to normal or sham adrenalectomized controls. On the other hand, the hypophysectomized animal consistently deposits more fat in the depot during a period of *ad libitum* feeding and moves fat out of the depot at a greatly reduced rate during a fast.

Stoerck and Porter (4), using large amounts of cortisone acetate (2 mg. daily) found that adrenalectomized animals showed a weight deficit but gained more adipose tissue. We used a relatively small dose of hydrocortisone (0.3 mg. daily) as well as a higher dose (1.0 mg. daily) and found that even at the lower dose range, this hormone prevented the net movement of fat out of the epididymal depot in normal and adrenalectomized animals in a 12-hour fasting period. The hypophysectomized animals without hormone treatment move so little fat out of this depot in a 12-hour fast that further inhibition would be seen only as an increase in fat deposition. There is some indication however, that under the circumstances of this complex hormonal deficiency (thyroxine, growth hormone, steroids) the administration of hydrocortisone at the low dose level may have augmented the movement of fat out of the depot. This is being investigated further. The work of Matthews *et al.* (9) suggested that there were marked metabolic differences in hypophysectomized animals kept for three months as compared to the acutely hypophysectomized preparation. We did not observe this in our procedures.

The behavior of the sham adrenalectomized animals with regard to depot fat changes was unanticipated. The adrenals were exposed, handled gently and the incision closed. Six days following this procedure, the animals were fasted and the usual depot fat measurements were made before and after the 12-hour fast. In more than 50 animals of this kind, it was found that there was a marked inhibition of the depot. The fasting, sham adrenalectomized animals behaved like the intact animals that had been pretreated for three days with hydrocortisone. The administration of hydrocortisone to the sham adrenalectomized preparation had no further effect on the rate of fat mobilization. We are investigating the time in-

terval between operation and fasting during which this effect persists in the sham adrenalectomized animal. In addition, the length of the fasting period is being increased to 24 hours in order to increase the caloric deficit and thus increase the stimulus to fat mobilization.

In a recent paper by Milstein and Coalson (10) it was reported that the trauma of body surface scalding, evoked an inhibition of fat mobilization from a peripheral depot. This stress is known to produce an augmented protein catabolism. We observed a similar "sparing" of fat breakdown in the presence of rapid protein mobilization in both normal and adrenalectomized animals treated with phlorizin (5). The data on sham adrenalectomized animals is consistent with these findings. The stress of the surgical procedure also appears to elicit the conditions which tend to inhibit fat mobilization while augmenting protein breakdown. These effects persist for at least six days following the application of the stress in our experiments and in those described by Milstein and Coalson (10). A similar metabolic pattern may be imposed by the administration of hydrocortisone but there is little evidence to show that the titers of circulating corticoids remain significantly elevated for long periods of time following relatively mild stresses. The inhibition of fat mobilization under these circumstances probably involves factors other than steroid titers in blood. These factors are obscure at this time but warrant further investigation.

The use of the epididymal fat depot as an index of total body fat changes is susceptible of valid criticism. These data do indicate, however, that this depot is affected directly or indirectly by an elevation or diminution of cortical steroids. The direction of these effects is to slow the rate of fat movement out of the depot when steroid titers are raised. The influence of hydrocortisone on the increase in appetite may have accounted for the increased deposition of fat in the depot during the three-day period of pretreatment. As indicated, however, there was no evidence of a significant gain in total body weight as compared to the untreated controls. This again suggests that both large and relatively small amounts of this hormone increase fat accumulation while simultaneously decreasing protein synthesis (4).

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RE-EVALUATION OF THE EVIDENCE FOR A PITUITARY ERYTHROPOIETIC HORMONE[1]

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ABSTRACT

Evidence for the erythropoietic potency of adrenocorticotrophic preparations in the absence of the adrenal has been reexamined. Pituitary fractions of known erythropoietic activity did not correct the anemia in hypophysectomized rats when the adrenal was also absent.

THE anemia which follows hypophysectomy in the rat and the correction of this anemia or production of polycythemia, by the administration of pituitary extracts shows that anterior pituitary hormones participate in the adjustment between the erythrocyte content of the blood and the oxygen requirements of the organism. This paper reviews the evidence which led this laboratory to postulate the existence of a pituitary erythropoietic factor distinct from the well known pitutitary hormones. A reappraisal of the evidence in the light of recent experiments, has led to the abandonment of this postulation.

White and Dougherty in 1945 (1) described stimulation of erythropoiesis in mice by pituitary adrenocorticotrophic preparations, and a joint communication from the Division of Medical Physics and this laboratory in 1951 (2) reported that adrenocorticotrophic preparations prevented the development of anemia in hypophysectomized rats, and elevated the red cell volume in intact rats above normal values. Subsequently, groups of workers from these laboratories reported that corticotrophic preparations increased red cell volume in the absence of the adrenals (3, 4).

The finding that corticotrophic preparations increased red cell volume in the absence of the adrenal could not be reconciled with the hypothesis that adrenocorticotrophic hormone (ACTH) is the pituitary erythropoietic factor unless one assumes that ACTH acts in the absence of its target organ. This assumption appeared to be unwarranted. If ACTH could act in the absence of its target organ one would anticipate, for example, that adrenalectomized rats (maintained on salt) would develop polycythemia, inasmuch as the adrenal stimulating potency of the pituitary and of the blood plasma is greatly increased after adrenalectomy (5); actually adrenalectomized rats develop a mild, transient anemia (6).

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In cross transfusion studies in which hypophysectomized rats were parabiotically united with normal rats, the adrenal weights of the hypophysectomized partners were not significantly greater than those of hypophysectomized controls (7, 8). This suggested that only minute amounts of ACTH were transferred through the capillary interchange. However, when one of a normal parabiotic pair was subjected to hypoxia the erythropoietic effect was demonstrable in the partner (9). The interpretation drawn from these experiments was that ACTH and the erythropoietic factor were substances having different survival times in the circulation (4). Implicit in this interpretation was the assumption that the erythropoietic factor was of pituitary origin.

There were other evidences that erythropoiesis is regulated by extra-adrenal mechanisms, such as the erythropoietic response of adrenalectomized rats to reduced barometric pressure, or the erythropoietic response of normal rats to hypoxia without increase in adrenal size (4, 6, 10). Hypophysectomized rats, though limited in their response to hypoxia, nevertheless respond with increased red cell volume. Yet the adrenals remain very small and inactive (4, 11). Though a small amount of adrenal function undoubtedly persists in hypophysectomized rats, there is no evidence that hypoxia, or any other stimulus, increases adrenal function in the absence of the pituitary.

In an effort to determine whether the pituitary erythropoietic stimulus could be traced to any of the known pituitary hormones, the most highly purified preparations available were assayed for their erythropoietic action. It was known that growth hormone converts the bone marrow of the hypophysectomized rat from a pale, fatty tissue to a red, cellular one; however this hormone did not ameliorate the anemia, judged by hemoglobin concentration or red cell counts (12), or prevent the development of of post-hypophysectomy anemia as judged by red cell volume when expressed in terms of final body weight (13). Growth hormone was therefore not identified as an erythropoietic stimulant; although the total red cell volume was increased, it was not increased to a degree sufficient to ameliorate the anemia in the face of the increased body size. Purified thyrotrophic hormone partially corrected the anemic condition in hypophysectomized rats (14). Highly purified lactogenic hormone, almost free of contamination with ACTH (15), when injected in daily doses of 50 to 1000 μg. for 14 days failed to increase the red cell volume in anemic hypophysectomized rats (16). Beta-melanocyte stimulating hormone, containing 1000 U./mg. (17) had no effect on the red cell volume of the anemic hypophysectomized rat when injected in daily doses of 100 μg. in the 14 day assay (18). An increase in erythropoiesis following injection of pituitary gonadotrophins has not been demonstrated, though a stimulation might be anticipated from injection of interstitial cell stimulants on the basis of the reported effects of androgens on blood formation.

The anemia in the hypophysectomized animal has frequently been attributed to the loss of the trophic hormones, and the consequent atrophy of the target organs. Amelioration of the anemia has in fact been described after administration of testosterone and thyroxine given alone, and in combination (19, 20). Adrenal cortical steroids have also been implicated and have been reported to increase the number of circulating red blood cells in normal rats (21), though not in hypophysectomized rats except when given in combination with thyroxine (22, 23).

Thus purified growth and thyrotrophic hormones had some effect on erythropoiesis though they did not increase the red cell volume of hypophysectomized rats to normal values, whereas adrenocorticotrophic fractions were effective not only in the repair of the anemia of hypophysectomized rats but also produced polycythemia in normal rats. Since adrenocorticotrophic fractions appeared to be effective in the absence of the adrenal it was considered improbable that the erythropoietic component in such pituitary fractions was ACTH, and a separate pituitary erythropoietic factor was postulated. Therefore efforts were continued to separate chemically the erythropoietic principle from ACTH. The chemical procedures which were found to concentrate the erythropoietic factor were always those most useful in the preparation of ACTH and no clear separation could be accomplished. However, the erythropoietic and adrenocorticotrophic potencies did not always parallel one another. For example, pituitary fractions prepared by autolytic digestion, by digestion with added enzymes, or by chromatographic methods (4, 24), increased red cell volume 20–40% yet cause no increase, or a barely perceptible increase, in adrenal weight and no evidence of increased adrenal function as judged by thymic weight. In most instances such preparations caused some depletion in ascorbic acid, or slight changes in lipid content of the adrenal.

When highly purified ACTH[2] was assayed and found to be a potent erythropoietic stimulant, this finding though damaging to the concept of the separate existence of a pituitary erythropoietic factor, was not accepted as definitive evidence due to the difficulties inherent in determining purity of pituitary hormones. Such difficulties are well known and often originate from the confusing multiplicity of biological effects reported for hormones considered pure by current physico-chemical criteria. The work factor (26), melanocyte expanding activity (17), the fat mobilizing factor (25, 27), and the specific metabolic factor (28, 29, 30) have been or still are associated with highly purified preparations of ACTH.

Meanwhile through the studies of Borsook (31), Van Dyke (32), Gordon

[2] Alpha-corticotropin was made available by C. H. Li and the assay here cited is referred to in his description of the biological properties of ACTH (25). Also tested were purified preparations of alpha-pool corticotropin from Lederle Laboratories, and corticotropin B from Merck and Co.

(33), Hodgson (34) and others, further knowledge has accumulated regarding erythropoietic factors in plasma and urine. Furthermore, Jacobson (35) and Crafts (36) and their collaborators have shown that such factors can be increased in the plasma of hypophysectomized animals by appropriate stimuli such as hypoxia or bleeding. The demonstration of a non-pituitary humoral factor invalidates the inference drawn from parabiotic studies regarding the separateness of the erythropoietic factor and ACTH. The erythropoietic response to hypoxia of the normal rat without adrenal hypertrophy, and of the adrenalectomized or hypophysectomized rat to this stimulus could be interpreted in terms of a similar extrapituitary humoral mechanism. The most cogent remaining evidence for the existence of a pituitary erythropoietic factor distinct from ACTH was the reported ability of pituitary fractions to stimulate erythropoiesis in the absence of the adrenal. The validity of this evidence has been re-examined.

In previous bio-assays of pituitary fractions in hypophysectomized-adrenalectomized rats difficulties had been encountered in maintaining the rats in good health, with resultant small numbers which survived the 14-day injection period. In efforts to determine a satisfactory regimen it had been found that substitution of 1% NaCl for drinking water was not adequate for maintenance after adrenalectomy combined with hypophysectomy. A larger proportion of the rats survived for the test period when given desoxycorticosterone acetate (DCA) in sesame oil, or desoxycorticosterone trimethylacetate in microcrystalline aqueous suspension. When DCA was combined with cortisol acetate the rats remained in even better condition. Due to the possible action of cortisol on body weight and red cell volume the latter steroid was not adopted for maintenance in all experiments.

Three series of experiments were performed in which corticotrophic preparations were tested for erythropoietic activity in hypophysectomized rats deprived of the adrenals. In the first two series, an oxycellulose eluate of sheep anterior pituitary was injected, in the third series alpha-corticotropin[3] was injected. In all series the preparations were assayed simultaneously in hypophysectomized rats.

The degree of anemia and its repair in hypophysectomized rats has been evaluated in this laboratory by determination of red cell volume using the Fe^{59} dilution method. This method though expensive and more time consuming than some other methods such as total red cell counts, reticulocyte counts, or hematocrit determinations, protects from errors introduced by possible changes in total blood volume. Due attention should be paid, however, to changes in body weight, or conditions which change the size or life span of the red cells (37). The test animal used in assay of erythropoietic potency of pituitary preparations was the female rat of the Long-Evans strain which was hypophysectomized at 26–28 days of age and used after a 45-day period. After this interval the post-hypophysectomy anemia has attained a reasonably stable value 40–50% below

[3] Alpha-corticotropin was kindly supplied by C. H. Li, of the Hormone Research Laboratory, Berkeley.

normal, the red cell volume (RCV) being on the average 1.40 to 1.50 ml./100 gm. BW. compared with the values in the normal rat of 2.30–2.40 ml./100 gm. BW. The pituitary fractions to be tested were suspended in beeswax and peanut oil and were injected subcutaneously daily for 14 days. Twenty-four hours after the last injection, red cell volumes were determined by the Fe^{59}-tagged red cell dilution method (38) which was briefly as follows: Labelled red cells were obtained from a rat previously injected with Fe^{59}. A small, known volume of blood containing Fe^{59}-tagged red cells of known specific activity was injected into the external jugular vein of the test animal under ether anesthesia. After 6 minutes to allow mixing of the injected sample in the recipient animal, a large sample of blood was drawn from the abdominal aorta into a heparinized syringe. The dilution of radioactivity was then determined in a blood sample of known volume. The red cell volume was calculated from these data and the hematocrit, and was then expressed in relation to body weight.

The oxycellulose eluate of sheep pituitary was prepared as follows. An aqueous extract of sheep pituitary was heated to 85° C. over an open flame with vigorous stirring

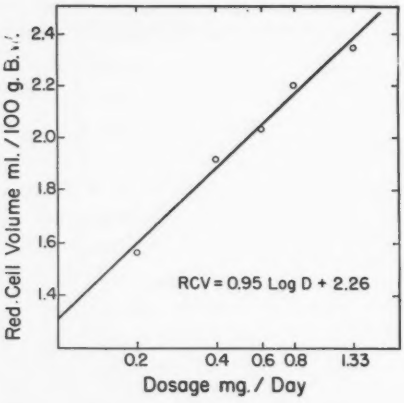

FIG. 1. Response of hypophysectomized female rats to graded doses of oxycellulose eluate. Each point represents the RCV of 15 to 30 rats.

The clear filtrate obtained from the coagulated suspension was brought to 0.1N by addition of glacial acetate acid (pH 3.8), was adsorbed on oxycellulose, eluted with 0.1N HCl, neutralized, dialyzed and lyophylized. The preparation was standardized carefully for erythropoietic activity in the anemic hypophysectomized rat before use in this series of experiments. The curve (Fig. 1) obtained from injection of increasing doses under standard assay conditions shows that the log dose-response relationship in hypophysectomized rats was linear.

The experimental animals were hypophysectomized at 28 days of age and injections were begun 51 days later. During this postoperative interval the rats were observed carefully, and any which appeared incompletely hypophysectomized (as judged by weight change, body tone, character of pellage or vaginal opening) were discarded. Rats which were also adrenalectomized were subjected to the second operation 50 days after hypophysectomy and injections were begun the next day. The adrenals when removed were carefully examined and no animals in which the cortex had been injured were included in experimental groups. At autopsy the completeness of hypophysectomy and adrenalectomy were determined by careful examination of the operation sites at 10-fold magnification. Any tissue not identifiable on gross examination was sectioned and stained appropriately. Cortical fragments less than 1 mm. in diameter were found in 6 of 62 adrenalectomized rats. Though the red cell volumes of these animals did not differ from those of completely adrenalectomized rats, these results were not included in the data

presented. The adrenalectomized rats were not subjected to the usual terminal test for completeness of adrenalectomy, i.e., transfer to a high potassium-low sodium diet (6) as the blood withdrawal necessitated in determinations of blood volume invalidates this test.

Table 1 shows that the corticotrophic preparations caused no repair of the anemia in hypophysectomized rats deprived of their adrenals, although these same preparations were effective in restoring to normal the red cell volume and other hematological values in the hypophysectomized rats which were injected concurrently. The hematological values in hypophysectomized-adrenalectomized rats receiving ACTH and supported by steroids were somewhat higher than in untreated hypophysectomized rats, but these values were also higher in hypophysectomized-adrenalectomized rats and in hypophysectomized rats injected only with steroids. The increase is therefore not attributable to the action of ACTH.

These experiments do not confirm the earlier reports, and give no support to the concept that the erythropoietically active pituitary component is a separate hormone. The substance traced in chemical fractionations of pituitary tissue by its effect on red cell volume in hypophysectomized rats appears to have been the adrenocorticotrophic hormone. Although it is well established that the pituitary plays a role in regulation of erythropoiesis, there is no satisfactory evidence for a pituitary erythropoietic factor distinct from the known pituitary hormones. The adrenocorticotrophic hormone is clearly instrumental in the regulation of red cell volume, as is thyrotrophic hormone and possibly growth and gonadotrophic hormones. The relative importance and mechanism of action of the several factors remains to be determined.

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TABLE 1. ERYTHROPOIETIC ACTIVITY OF SHEEP OXYCELLULOSE ELUATE AND ALPHA-CORTICOTROPHIN IN HYPOPHYSECTOMIZED-ADRENALECTOMIZED ($\overline{A}\overline{H}$) AS COMPARED WITH HYPOPHYSECTOMIZED RATS

Series	Group	Age At			Maintenance‡	No. of rats	Body weight, gm.		Hematocrit %	Hemoglobin, gm./100 ml.	Total blood volume, ml.	Red cell volume, ml./100 gm. BW	Adrenals, mg.	Thymus, mg.
		$\overline{H}$ da.	$\overline{A}$ da.	Onset inj., da.			Initial	Final						
I*	$\overline{HA}$ Inj.	28	78	79	DCA 50 μg./da.	5	82	91	31.4±1.4	9.4±0.2	4.13±0.34	1.43±0.14	—	121
	$\overline{HA}$ Cont.	28	78	—	DCA 50 μg./da.	7	82	84	29.1±1.1	9.1±0.3	4.25±0.17	1.47±0.08	—	109
	$\overline{H}$ Inj.	28	—	79	—	5	78	91	42.6±1.4	12.4±0.5	4.93±0.25	2.31±0.07	21	<25
	$\overline{H}$ Inj.	28	—	79	DCA 50 μg./da.	5	77	80	42.3±5.2	10.7±1.2	4.61±0.32	2.44±0.39	29	<25
	$\overline{H}$ Cont.	28	—	—	—	5	79	88	24.1±2.1	7.7±0.7	4.04±0.14	1.11±0.11	8	113
II*	$\overline{HA}$ Inj.	28	78	79	DCA 50 μg./da.	2	75	73	33.9±0.2	11.0±0.0	3.65±0.59	1.70±0.05	—	87
	$\overline{HA}$ Inj.	28	78	79	Cortisol 50 μg./da. DCA 50 μg./da.	8	75	81	36.4±1.5	11.5±0.5	4.21±0.22	1.89±0.06	—	73
	$\overline{HA}$ Cont.	28	78	—	DCA 50 μg./da.	3	77	77	28.1±1.5	9.5±0.2	3.96±0.10	1.45±0.07	—	68
	$\overline{HA}$ Cont.	28	78	—	Cortisol 50 μg./da. DCA 50 μg./da.	9	73	77	34.3±1.1	10.6±0.4	3.90±0.15	1.74±0.07	—	63
	$\overline{H}$ Inj.	28	—	79	—	5	72	82	40.8±0.8	12.1±0.3	4.77±0.21	2.37±0.17	25	<25
	$\overline{H}$ Cont.	28	—	—	—	5	74	80	27.8±0.8	9.5±0.2	4.08±0.26	1.42±0.04	7	102
III†	$\overline{HA}$ Inj.	28	75	76	DCA (trimethyl) 1.25 mg. Cortisol 50 μg./da.	11	74	75	39.0±1.4	12.4±0.5	3.45±0.09	1.79±0.07	—	57
	$\overline{HA}$ Cont.	28	75	—	DCA (trimethyl) 1.25 mg. Cortisol 50 μg./da.	11	72	77	36.1±1.3	11.3±0.4	3.93±0.14	1.84±0.06	—	58
	$\overline{H}$ Inj.	28	—	76	—	5	70	78	40.8±2.3	11.5±0.3	4.58±0.21	2.40±0.16	31	<25
	$\overline{H}$ Cont.	28	—	—	—	5	71	81	30.2±1.5	9.4±0.3	3.88±0.17	1.45±0.06	8	108

* Injected groups received 1.3 mg. oxycellulose eluate suspended in 0.1 ml. of 4% beeswax in peanut oil subcutaneously, daily for 14 days.
† Injected groups received 25 μg. alpha-corticotrophin suspended in 0.1 ml. of 5% beeswax in peanut oil subcutaneously, daily for 14 days.
‡ Desoxycorticosterone acetate (Ciba) was given in a 0.05 ml. sesame oil subcutaneously, daily for 14 days.
Desoxycorticosterone trimethyl acetate (Ciba) was given in a single subcutaneous injection in 0.9% NaCl on the day of adrenalectomy.
Cortisol (Merck hydrocortone acetate) was given in 0.1 ml. of 0.9% NaCl subcutaneously daily for 14 days.

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CONVERSION OF C^{14}-TESTOSTERONE TO C^{14}-ESTROGENIC STEROIDS BY ENDOCRINE TISSUES[1]

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ABSTRACT

The ability of stallion testis, human term placenta and a human feminizing adrenal cortical carcinoma to convert C^{14}-testosterone to C^{14}-labeled estrogenic steroids has been demonstrated. The relationship between this conversion and the biosynthesis of estrogens by these tissues has been discussed.

STUDIES previously reported from these laboratories demonstrated the conversion of testosterone-3-C^{14} to C^{14}-estradiol by human ovarian slices (1). Similar results have been obtained by Wotiz, *et al.* who reported the formation of C^{14}-labeled estrone and estriol as well as estradiol from C^{14}-testosterone incubated with human ovarian tissue showing cortical stromal hyperplasia (2). In addition to these studies Heard, *et al.* have found that estrone-C^{14} is a urinary metabolite of testosterone-4-C^{14} in the pregnant mare (3). Meyer has shown the formation of estrone from 19-hydroxy-4-androstene-3,17-dione in endocrine tissue and has suggested that the 19-oxygenated compound is an intermediate between the C_{19} neutral steroids and C_{18} aromatic steroids (4).

There is no direct evidence that this pathway from testosterone to estrogens is the major one operating in the endocrine tissues producing estrogens. Some further indirect evidence bearing on this point can be

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furnished by studies of the ability of various tissues to carry out the conversion of testosterone to estrogenic steroids.

The results presented in this paper demonstate the ability of three additional types of endocrine tissues, all thought to produce estrogens, to carry out the conversion. These tissues are stallion testis, a human adrenal carcinoma, and a human placenta.

EXPERIMENTAL

Counter-Current Distributions

All 49- and 99-transfer counter-current distributions were carried out in a 100-tube glass Craig counter-current distribution apparatus (H. O. Post Scientific Apparatus Company). Unless otherwise indicated, equal volumes, 10 ml. each, of upper and lower layers were used for each transfer. The fractions were evaporated under reduced pressure with constant agitation, or from the frozen state.

Short distributions, four or eight transfers, were carried out in separatory funnels, lower layers being transferred.

Determination of Radioactivity

Samples for radioactivity analysis were plated directly in stainless steel counting dishes (1 inch in diameter, $\frac{5}{16}$ inch in depth) from organic solvents, usually 95% ethanol. All samples were essentially infinitely thin, less than 0.3 mg./cm.^{2} They were counted in a windowless flow counter. At least 2000 counts were accumulated and all counts reported are corrected for background.

Determination of Estrogens

The counter-current distributions were analyzed for estrogen by the method of Engel *et al.* (5), measuring fluorescence at 488 mμ after heating in H_2SO_4 (6). A Farrand photofluorometer was utilized for the measurement of fluorescence.

Tissue Incubations and Extractions

Stallion Testis. A single testis (65 gm.) removed from a four-year old Palomino[5] was immediately homogenized for two minutes in a Waring blendor with 120 ml. of 0.85% NaCl solution. The homogenized mixture was centrifuged at low speed and most of the supernatant fluid was decanted. The residue was suspended in 50 ml. of 0.06 M phosphate buffer (pH 7.4) containing 0.075 mM of sodium fumarate, 0.75 mM of nicotinamide, and 0.5 mM of $MgCl_2$. The homogenized tissue was divided equally among five 125 ml. Erlenmeyer flasks each containing 1 mg. of testosterone-3-C^{14} (490,000 cpm/mg.) dissolved in 0.1 ml. of propylene glycol. Incubation was carried out at 37° for 3 hours with shaking, using air as the gas phase.

The contents of the five flasks were combined and 5.0 mg. of estrone were added. The mixture was dried from the frozen state and the residue extracted in a Soxhlet extractor as described by Ryan and Engel (7).

Adrenal Carcinoma. This tumor, which weighed 2 Kg., was removed from a 66-year old male showing marked gynecomastia. His preoperative urinary 17-ketosteroid level was 510 mg./24 hr. (8). Paper chromatographic (9) examination of the extract disclosed a high proportion of dehydroepiandrosterone. Excretion of estrogens was also markedly elevated. The daily excretion of estrone was 187 μg.; estradiol, 47 μg.; and estriol, 170 μg.

[5] We wish to thank Mr. Dwight Jacobs, Lynbrook Farm, Southborough, Massachusetts, for his cooperation in making this tissue available.

(5). Levels of 17-ketosteroids and estrogens fell to low levels post-operatively, but subsequently increased with growth of metastases.

A portion of the tumor was sliced in a Stadie slicer (0.5 mm. thickness) and 50 gm. of slices were added to each of two 750 ml. Erlenmeyer flasks containing 100 ml. of high potassium medium (10), 300 mg. glucose, 0.1 mM of potassium fumarate, and 0.23 mg. of testosterone-3-C^{14} (2.35×10^6 cpm/mg.) in 0.2 ml. of propylene glycol. The flasks were flushed with 95% O_2 – 5% CO_2 and incubated with shaking at 37° for 3 hours. Ten mg. each of estrone, estradiol, and estriol as well as several neutral steroids were added to the incubated mixture which was then homogenized in a Waring blendor and extracted with a mixture of methanol and chloroform according to a modification of the method of Folch (11).

Human Placenta. Tissue from a human term placenta was sliced in a Stadie slicer (0.5 mm. thickness), and 7.5 gm. of slices were incubated in 18 ml. of human serum enriched with 0.018 mM of potassium fumarate and 10 mg. of glucose and containing 0.23 mg. testosterone-3-C^{14} (2.35×10^6 cpm/mg.) previously dissolved in 0.2 ml. propylene glycol. The flask was flushed with 95% O_2 – 5% CO_2 and incubated 3 hours at 37° with shaking.

Two mg. each of estrone, estradiol and estriol were added to the incubation mixture as carriers along with 10 mg. each of several neutral steroids. The mixture was homogenized in a Waring blendor and extracted repeatedly with ethyl acetate, and ether:chloroform (4:1), and then with 10 volumes of acetone (12). Finally the residue was extracted three times with 20 volumes of chloroform:methanol (2:1). The combined extracts were evaporated to dryness. The residue was dissolved in chloroform and washed with 1/10 volume of water.

Preliminary Purification of Crude Extracts

Each of the extracts was subjected to an 8 transfer counter-current distribution between hexane and 90% methanol. In each case there was a peak in radioactivity in funnel 8 which contained the most polar material. In each experiment the contents of funnels 5–8 contained the bulk of the radioactivity and the carrier steroids present in the crude extract and were therefore combined. These polar fractions were next subjected to phenolic separations. In the case of the material from the incubations with stallion testis and the adrenal tumor, phenolic separations using toluene and IN NaOH were carried out (5), followed by 8-transfer distributions in the same solvent system (1). In both cases there were peaks in activity in the NaOH ends of the distributions. In the case of the fraction from the incubation with placental tissue, the phenolic material was separated by passing the fraction dissolved in methanol through an ion exchange resin (Nalcite SAR-8) in the bicarbonate cycle and washing the resin with additional methanol to remove neutral material.[6] The phenolic material was then eluted with 5N acetic acid in methanol. This procedure was repeated on the phenolic fraction from the first column.

The phenolic fraction from the incubation of stallion testis contained 2410 cpm; the one from the incubation of the adrenal carcinoma, 9730 cpm; and the one from the incubation of placenta, 316,000 cpm.

Separation and Purification of Estrogenic Steroids

Stallion Testis. Two mg. of estradiol were added to the phenolic material and the mixture subjected to a 99-transfer counter-current distribution in 70% methanol-30% H_2O/40% $CHCl_3$—60% CCl_4.

[6] Engel, L. L. Unpublished data.

There was a small peak in radioactivity in the same tube as the peak in estrone ($K^7 = 0.21$); however, the level of radioactivity was too low to permit accurate measurement or further study. A peak in radioactivity also coincided with the peak in estradiol ($K = 1.04$); however, this region was contaminated by radioactivity slightly more polar than the estradiol. The contents of the tubes containing the estradiol (1.88 mg.) were pooled and subjected to a 49-transfer counter-current distribution in a solvent system consisting of 30% ethyl acetate—70% cyclohexane/50% ethanol—50% water.

Statistical analysis of the second counter-current distribution in the estradiol region for 1.5θ tubes (13) indicated radiochemical purity, the p value being greater than 0.2 and the coefficient of variation for specific activity, 0.11 (14). However, the counter-current distribution curve indicated that a more polar impurity overlapped the estradiol slightly. The specific activity was 400 ± 44^8 cpm/mg.

Adrenal Carcinoma.—The phenolic fraction was subjected to a 99-transfer counter-current distribution in 30% ethyl acetate—70% cyclohexane/50% ethanol—50% H_2O. Peaks in radioactivity coincided with the peaks in estrone and estradiol. The mean specific activity of the estrone ($K = 1.75$) in the region of the peak was 1260 ± 40 cpm/mg. The estradiol peak ($K = 0.78$) had a mean specific activity of 864 ± 21 cpm/mg. There was no peak in radioactivity associated with the peak in estriol ($K = 0.053$).

Placenta. The phenolic material was subjected to a 99-transfer distribution in 70% methanol—30% H_2O/40% $CHCl_3$—60% CCl_4. A peak in radioactivity conicided with the peak for estrone ($K = 0.22$). It contained 0.40 mg. of estrone and 82,300 cpm. The experimental points for radioactivity did not fit the theoretical distribution curve, indicating a lack of purity. The estradiol peak also corresponded to a peak in radioactivity ($K = 1.02$). This peak contained 0.42 mg. of estradiol and 79,100 cpm. As in the case of the estrone, the estradiol was not radiochemically pure. There was considerable radioactivity in a peak slightly more polar than the peak in estriol ($K = 4.82$). This radioactivity overlapped the estriol peak, thus obscuring any peak that might have coincided with the estriol. The contents of tubes 0–64, containing all the estrone and estradiol, were combined; contents of the remaining tubes, 65–99, containing the estriol and the polar radioactive compound, were also combined.

To the pool containing the estrone and estradiol from the preceding distribution was added an additional 10 mg. each of estrone and estradiol. The mixture was then subjected to a 170-transfer counter-current distribution in a solvent system consisting of 70% methanol—30% H_2O/20%

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[7] Partition coefficient, K = (concentration in upper phase)/(concentration in lower phase).

[8] Standard deviation.

$CHCl_3$—80% CCl_4. Peaks of radioactivity were associated both with the peak of estrone and that of estradiol; however, neither compound was yet radiochemically pure.

The estrone from the distribution of estrone and estradiol was subjected to a 99-transfer counter-current distribution using 40% ethyl acetate—60% cyclohexane/60% ethanol—40% water. A single peak of radioactivity and fluorescence (K = 1.02) was obtained. Statistical analysis of the data indicated radiochemical purity of this substance. The estrone had a specific activity of 5041 ± 625 cpm/mg.

The estradiol region from the 170 transfer counter-current distribution of the combined estrone and estradiol was pooled. This material was subjected to a 99-transfer distribution in 30% ethyl acetate—70% cyclohexane/50% ethanol—50% H_2O. A single peak of radioactivity and fluorescence was observed (K = 0.71). Statistical analysis indicated radiochemical purity. The estradiol had a mean specific activity of 4375 ± 644 cpm/mg.

The estriol from the first distribution was diluted with an additional 10 mg. of unlabeled estriol and subjected to a 99-transfer counter-current distribution between 50% ethyl acetate—50% cyclohexane and 33% ethanol—67% H_2O. The peak in estriol was associated with a much broader peak in radioactivity. The contents of the tubes around the peak of estriol were pooled. These contained 65% of the total amount of carrier estriol in the peak. This estriol was subjected to a third counter-current distribution. Ninety-nine transfers were applied in a solvent system consisting of 70% methanol—30% H_2O/60% $CHCl_3$—40% CCl_4. The volumes of the upper phases were 5 ml. for each tube, one-half the volume of the lower phase. The peak in estriol (K = 2.60) appeared to be associated with a peak in radioactivity. However, a second peak in radioactivity (K = 1.60) overlapped the peak in estriol, thus preventing an evaluation of its radiochemical purity.

The tubes containing 95% of the carrier estriol were combined and subjected to a 250 transfer counter-current distribution with recycling in 70% methanol—30% H_2O/60% $CHCl_3$—40% CCl_4, using a phase volume ratio of 0.5. This separated the less polar contaminant from the carrier estriol. However, statistical analysis of the estriol region indicated that the radioactivity has been separated from the carrier estriol and that the displacement of the two peaks was 1.4 tubes.

In order to substantiate this observation, the material in the region containing the carrier estriol was methylated by the procedure of Brown (15) and the product was subjected to a 99-transfer distribution, using 50% methanol—50% H_2O/carbon tetrachloride as solvent. The carrier estriol methyl ether had a partition coefficient of 1.3 and the radioactivity

a partition coefficient of 2.3, thus confirming the small separation observed in the original 250 transfer distribution.

DISCUSSION

The data presented herein demonstrate the ability of three endocrine tissues to carry out the conversion of testosterone to estrogenic steroids. The ability of ovarian tissue to produce estradiol from testosterone has been reported previously (1, 2). Although markedly different in origin and structure, these tissues are all considered capable of producing estrogens *in vivo*.

Stallion testes have been found to contain a very high concentration of estrogenic steroids (16). It is assumed that the estrogens in stallion urine (17, 18, 19) are metabolites of the estrogen produced by the testicular tissue. The experiment presented here indicates that the testicular estrogen may have as its precursor testosterone or a similar nineteen-carbon steroid produced by the testis. Estrogen has also been demonstrated in human testicular tissue, both normal (20) and neoplastic (21), and it is reasonable to suppose that its mode of formation is similar.

The conversion of testosterone to estrogenic steroids by the adrenal carcinoma is a finding consistent with the evidence indicating that such tumors are capable of producing estrogens *in vivo*. The patient in this study had gynecomastia and loss of libido, both signs of overproduction of estrogens. In addition, his urinary excretion of estrogens was markedly elevated prior to the removal of the tumor. Since the conversion of testosterone to estrogenic steroids has been demonstrated in patients lacking adrenals and gonads (22) and since this patient was excreting large amounts of C_{19} steroids, the possibility exists that the urinary estrogens were metabolites produced in non-endocrine tissue from the compounds secreted by the tumor. Another possibility is that the tumor secreted some substance, perhaps a trophic hormone, which stimulated the secretion of estrogens by the unaffected adrenal or the gonads. The demonstration that the tumor tissue was capable of converting testosterone to estrogenic steroids seems to indicate that the enzymes required for the terminal steps in one pathway for the formation of estrogens were present. This, in conjunction with the evidence from the urinary excretion studies that the tumor produced C_{19} steroids, is strong suggestive evidence that a conversion of this type could be responsible for the production of estrogenic steroids. In males, tumors of the adrenal cortex are often associated with feminization (23). Salhanick and Berliner (24) have recently described the isolation of equilenin from such an adrenal tumor. That the normal adrenal produces estrogens is indicated by the isolation by Beall (25) of estrone from beef adrenals.

In a parallel study in which slices of this adrenal tumor were incubated with 50 μC of CH_3 $C^{14}OO$ Na, no measurable radioactivity was found associated with the estrogens isolated,[9] demonstrating that under the conditions used testosterone was a far better precursor than acetate. In the case of the incubation with C^{14} acetate, however, no labeled neutral steroids could be isolated, indicating either that the amount of C^{14} acetate was insufficient or the conditions unsatisfactory for appreciable incorporation of acetate into the steroids.

The third experiment demonstrates the ability of placental tissue to synthesize estrogenic steroids from testosterone and is in accord with the observations of Ryan who demonstrated aromatization of testosterone by human placental microsomes in the presence of reduced triphosphopyridine nucleotide and molecular oxygen (26). The human placenta has been well established as a source of estrogens both by direct isolation (27, 28, 29) and by the effect of its presence on urinary estrogens (30). In addition, dog placenta was shown by Meyer to convert 4-androstene-3,17-dione and 19-hydroxy-4-androstene-3,17-dione to estrone (4).

These experiments together offer further suggestive evidence that testosterone or a similar nineteen carbon steroid is a normal intermediate in the biosynthesis of estrogenic steroids. Although these experiments are not strictly comparable since the incubation conditions were different, the differences in the extents of conversion of testosterone to estrogens are striking. The relatively small conversion of testosterone by the stallion testis is consistent with the fact that the predominant function of testis is to secrete C_{19} steroids. The low conversion by the adrenal tumor fits well into the over-all nature of its secretion as judged by urinary steroids. In this respect the tumor is similar to the stallion testis. Although the effects of the estrogens on the male host were marked, estrogens were quantitatively minor products compared with the C_{19} steroids.

The striking conversion of testosterone to estrogens by the placenta is of particular interest. If testosterone is an intermediate in the synthesis of estrogens by this tissue, it must undergo rapid turnover since there is no evidence suggesting that the placenta releases significant quantities of androgens into the blood stream. The enzymatic reaction limiting the production of estrogen is more likely to be one involved in the synthesis of testosterone rather than in the conversion of testosterone to an estrogenic steroid. It is interesting to consider the possible role of testosterone as a precursor of estrogens in the placenta in the light of other steroids produced by this organ. Progesterone, a well-established hormone of the placenta, has been shown to be oxidized to 17α-hydroxyprogesterone by testicular and ovarian tissue (31, 32, 33, 34). The ability of the placenta to form 17α-hydroxyprogesterone has been indicated by the work of Herr-

[9] Engel, L. L. and B. Baggett. Unpublished data.

mann (35) who found an increase in urinary excretion of pregnane-3α, 17α, 20α-triol in pregnancy. The conversion of 17α-hydroxyprogesterone, in turn, to C_{19} steroids has been demonstrated in testicular tissues (32), although not in placental tissue. The steps by which placental progesterone may be converted to placental estrogens are, therefore, nearly all established. This interesting relationship between the two classes of steroidal female sex hormones was suggested earlier by Solomon *et al.* (34) with reference to the ovary, and more recently supported by the experiments of Savard *et al.* (36) with the pregnant mare.

The high activity of the placenta with respect to steroid metabolism is further attested by the presence of as yet unidentified phenolic transformation products of testosterone. Attention is drawn in particular to the polar substance which followed the carrier estriol with such tenacity and which were separated only after a 250 transfer counter-current distribution and methylation. These findings emphasize once more the need for the application of stringent criteria of radiochemical purity before concluding that a radioactive transformation product and an added carrier are identical.

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NOTES AND COMMENTS

THE EFFECTS OF NEUROHYPOPHYSIAL EXTRACTS ON THE TADPOLE OF THE FROG, *HELEIOPORUS EYREI*

ABSTRACT

Heleioporus eyrei tadpoles increase their body weight when injected with Pitocin but not Pitressin. This response takes place at a relatively early stage of development compared to the other species previously tested.

Adult Anura respond to injections of neurohypophysial extracts by increasing their body weight. This is due to an increase in the rate of water uptake through the skin and an antidiuresis (1). This response has not yet been demonstrated in marine and freshwater gill-breathing species (2) with the notable exception of the axolotl (3). The tadpoles of Anura are in an interesting position in that they are a fresh water form that breathes by mean of gills, destined to become a terrestrial species with all the associated problems of water balance. The tadpole of the toad *Bufo bufo* does not respond, by increasing its body weight, to injection of neurohypophysial hormone till it has reached the stage of development where its tail disappears (4). Whether all larval Anura respond at such a relatively late stage of development is not known but it appeared possible that it may develop earlier in a species that will live in drier conditions than *Bufo bufo*.

The tadpoles of the burrowing frog *Heleioporus eyrei* which lives in a large area in southwest Australia were studied.

Injections of Pitressin and Pitocin (Parke, Davis & Co.) were given with an "Agla" micrometer syringe. The skin at the base of the tail was punctured and the needle pushed under the skin to the center of the back where the injection was made. The hormones were given in distilled water (1% of the body weight). The animals ranged in weight from 1.5 to 2.8 gm. and were matched for size and state of development in two groups. Hind limb rudiments were visible but the fore limbs were not due to appear for several weeks. The animals were weighed to 0.1 mg. in a tared weighing bottle containing tap water. During the experiments the animals were kept in tap water at 16 to 18° C and not fed.

With Pitressin 1 U./100 gm. there was a loss in weight over a 3 hr. period of 0.61 gm./100 gm. (S.E. $\pm$0.92) in 18 animals while with the same dose of Pitocin there was an increase in weight of 4.01 gm./100 gm. (S.E. $\pm$0.82) in 16 animals. This difference in response is highly significant ($t = 3.696$ $P < 0.001$). Thus these tadpoles show a differential response to Pitocin and Pitressin, the former being active in increasing the body weight as found in adult *H. eyrei* (5). Whether this increase in weight is due to an antidiuresis and/or an increase in water uptake through the skin is unknown but the definitive mesonephric kidney takes up trypan blue which indicates that it is functional (6).

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EFFECT OF HYPOPHYSECTOMY AND GROWTH HORMONE ADMINISTRATION UPON THE GOLGI APPARATUS IN THE ISLETS OF LANGERHANS IN THE RAT

ABSTRACT

The histology of the islets of Langerhans of the hypophysectomized rat was studied using the state of the Golgi apparatus as a criterion of activity. This seems to be a somewhat more sensitive indicator than the general stains usually employed. Twelve male Long-Evans rats were hypophysectomized at 40 days of age and sacrificed 14 days post-operatively along with a group of 12 normal pair-fed controls, 6 normal *ad libitum*-fed controls and 4 animals fed *ad libitum* and given 2.5 mg. of growth hormone per day intraperitoneally for 4 days. Slides were prepared for demonstration of the specific granules and the Golgi apparatus. In none of the groups were significant changes noted in the granules of either alpha or beta cells, but in the Golgi preparations there was striking atrophy of the beta cell Golgi bodies in the hypophysectomized animals as compared with either the *ad libitum*- or the pair-fed controls. Treatment with growth hormone restored the Golgi apparatus toward normal, but the significance of this observation remains in doubt because the food intake was not restricted. The alpha cell Golgi bodies were not altered in any of the groups.

Since it was observed that administration of large amounts of anterior pituitary extracts would produce permanent diabetes in the dog, considerable work has been devoted to elucidating the normal relationships between the pituitary and the islets of Langerhans. A trophic relationship between the pituitary and the beta cells of the islets similar to that existing between the pituitary and other target organs was postulated by Anselmino and coworkers (1, 2). It has been since confirmed that anterior pituitary extracts (3), and particularly growth hormone (4), will produce islet hyperplasia. Nevertheless, the idea of a trophic relationship has been discarded because hypophysectomy does not result in atrophy of the beta cells similar to that found in the target organs for which such a relationship has been established. Furthermore, there is no evidence indicating that the ability of the pancreas to produce insulin is impaired.

The relationship between the anterior pituitary and the alpha cells of the islets has only recently been explored and is clouded by conflicting evidence. Ferner, *et al.* (5) and Kracht (6) have claimed that hypophysectomy reduces the ratio of alpha to beta cells and that administration of growth hormone restores the normal ratio. Their studies were based upon silver stains for demonstration of the alpha cells, a procedure which has been called into serious question by Volk, Goldner and Frank-Crowley (7). These authors have pointed out the capricious nature of silver preparations and have restudied the islets of hypophysectomized rats using several other stains which they felt would demonstrate the alpha cells more reliably. They were unable to find evidence of atrophy of the alpha cells, nor changes following growth hormone administration except after very prolonged treatment. Under these conditions, the number of alpha cells initially increased and then decreased. Other investigators have found either no change in the alpha cells after growth administration (8) or an increase in activity (9).

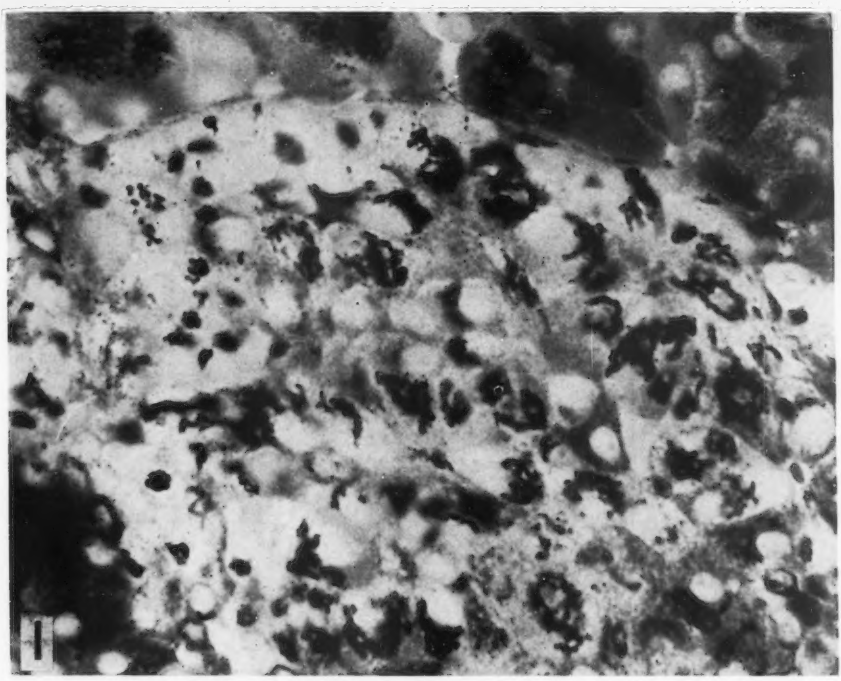

FIG. 1. Golgi preparation of islet of normal *ad libitum*-fed rat. Note preponderance of beta cells, centrally located, with large, elaborate Golgi bodies. Left pole of islet shows several alpha cells with typical clear cytoplasm and small Golgi bodies. ×917.

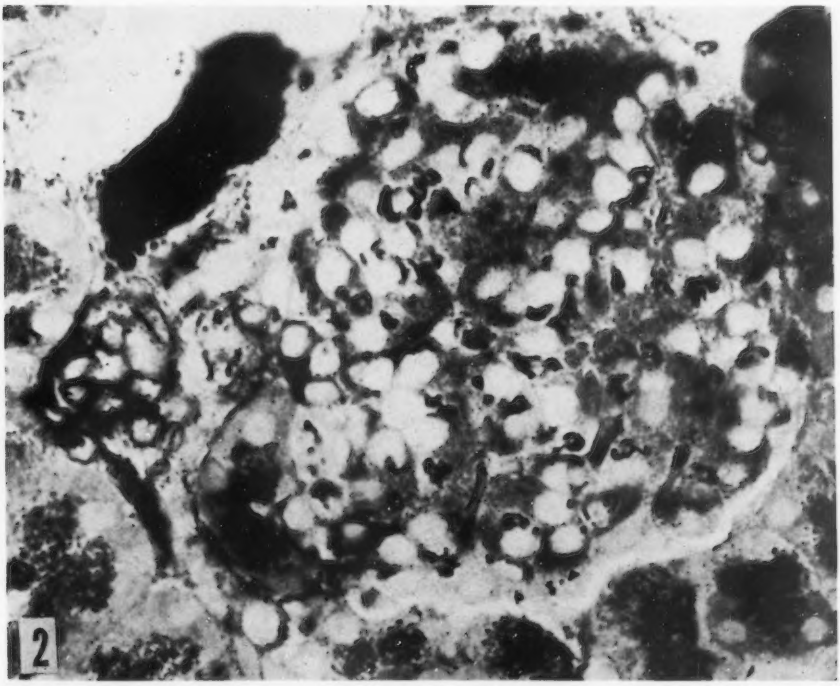

FIG. 2. Golgi preparation of islet from a hypophysectomized rat. Islet consists almost entirely of beta cells with very small Golgi bodies. ×917.

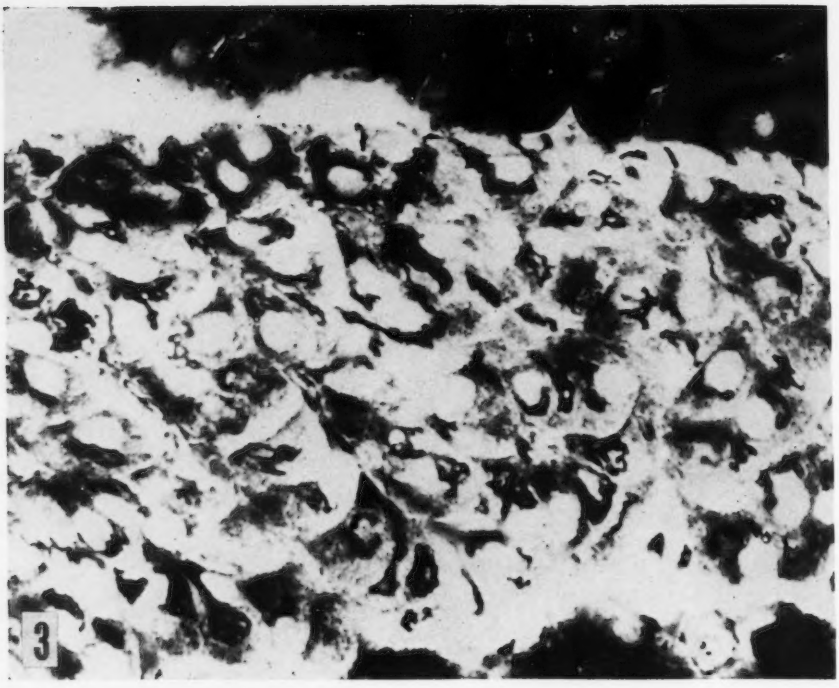

FIG. 3. Golgi preparation of islet from a control rat pair-fed with the animal whose islet is seen in Figure 2. Islet consists mostly of beta cells with much more elaborate Golgi bodies than seen in Figure 2. ×917.

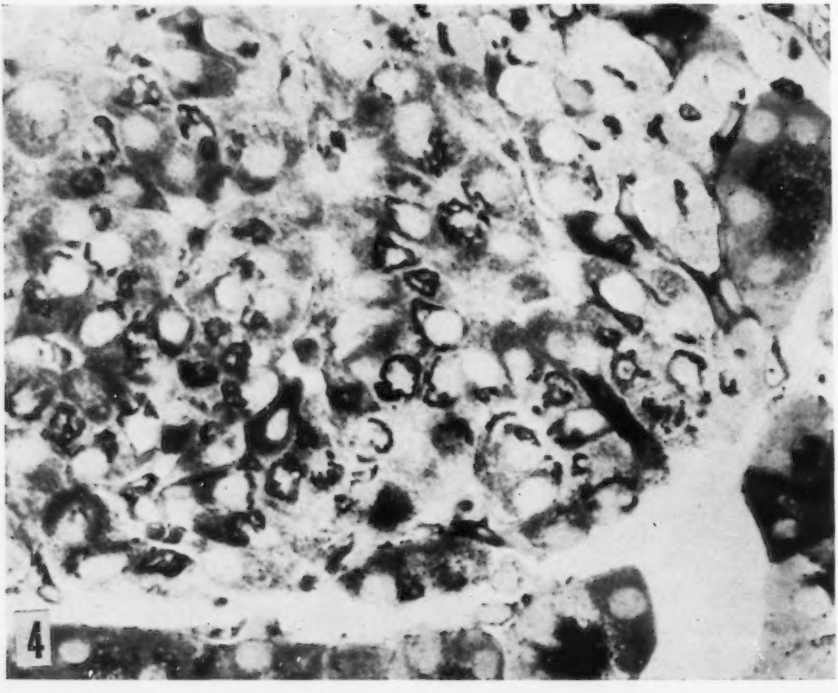

FIG. 4. Golgi preparation of islet from growth hormone-treated rat. Beta cell Golgi bodies are more elaborate than those of hypophysectomized rat in Figure 2. ×917.

Enough uncertainty still remains about the effects of altered levels of pituitary function on the alpha and beta cells that it seemed worthwhile to restudy the islets of rats after hypophysectomy and after growth hormone administration, using as a criterion of activity the state of the Golgi apparatus.

METHODS

Twelve male Long-Evans rats were hypophysectomized by the parapharyngeal approach at 40 days of age and were then placed in individual cages so that the daily food intake could be measured. Twelve normal rats of the same sex and age were also placed in individual cages at this time and each was pair-fed with one of the hypophysectomized rats. Both groups were sacrificed after 14 days. Completeness of operation was ascertained at autopsy by examination of the operative site using a binocular dissecting microscope. A group of 4 hypophysectomized female rats of 40 days of age was given 2.5 mg. of growth hormone per day intraperitoneally, with food allowed *ad libitum*. These animals were sacrificed after 4 days of treatment together with a group of normal controls also fed *ad libitum*. Other animals treated with growth hormone were restricted to the food intake of hypophysectomized animals, but unfortunately, these animals died, presumably of hypoglycemia, as described by Milman and Russell (10). In all cases, part of the tail of the pancreas was fixed in Bouin's fluid, cut at 4 μ and stained with Gomori aldehyde fuchsin, chromotrope and light green for specific granules, while another part of the tail of the pancreas was fixed in Champy-Kull fixative and the Golgi apparatus impregnated by the Nassonov-Kolatchev method. The growth hormone employed was prepared by the method of Wilhelmi *et al.* (11).

RESULTS

Preparation stained for specific granules. The islets of the normal animals showed well-granulated beta cells and the usual shell of moderately granulated alpha cells. The beta cells in the hypophysectomized animals appeared to be somewhat smaller but contained a normal amount of granular material and looked normal otherwise. The alpha cells were unchanged. The islets of the pair-fed animals were indistinguishable from those of the normal controls.

Golgi preparations. As described previously (12), the two cell types in normal islets showed Golgi bodies of very different appearance. Those of the beta cells were quite large and moderately elaborate while those in the alpha cells were quite small and simple (Fig. 1).

In the beta cells of all of the hypophysectomized animals the Golgi bodies were extremely small and simple (Fig. 2). Those of the alpha cells were apparently unchanged.

The animals which were pair-fed with the hypophysectomized animals showed some reduction in the intricacy of their beta cell Golgi bodies (Fig. 3), but this never approached the degree of change observed in the hypophysectomized animals. The alpha cells appeared to be unchanged when compared with either the hypophysectomized or the normal *ad libitum*-fed controls.

The animals treated with growth hormone showed a definite increase in the size and complexity of the beta cell Golgi bodies and no change whatever in those of the alpha cells (Fig. 4).

DISCUSSION

The basis for using the appearance of the Golgi apparatus as a criterion of the functional state of the islet cells has been discussed in a preceding paper (12). If the interpretation of the changes in the Golgi bodies in the hypophysectomized animal is accepted as indicating a reduction in function, then it is necessary to consider the significance of this change. The explanation could lie along at least two lines. First, the hypophysectomized animals may have had lower blood sugars than did the pair-fed controls, but it is highly unlikely that this was the case, since it is well-established that the hypophysectomized animal allowed food *ad libitum* maintains normal blood glucose levels (13). Second, the diminution in activity might have resulted from removal of one or more pituitary hormones which ordinarily directly stimulate the beta cells. This does not necessarily imply a trophic relationship, however, since the hypophysectomized animal obviously retains a

considerable ability to produce insulin. The question of whether or not the beta cells of the hypophysectomized animal are able to respond normally to the stimulus of elevated glucose levels is currently under investigation.

It is not possible at this time to decide which of the pituitary hormones may be involved, since at least ACTH, lactogenic and growth hormone are considered to act as stimuli to the beta cells either directly or indirectly. The observation that growth hormone appeared to restore the Golgi bodies of the beta cells toward normal would tend to favor the possibility that it, at least, is involved. However, it has not yet been possible to separate its effect from changes in food intake, inasmuch as our group of growth-hormone-treated animals on restricted food intake died.

The absence of any change in the appearance of the Golgi apparatus in the alpha cells is another example of their curious constancy of appearance in the face of what are supposed to be stimuli to the release of glucagon.

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ALKALINE PHOSPHATASE ACTIVITY IN THE PITUITARY BODY OF THE RAT

ABSTRACT

The posterior lobe of the pituitary body of the rat has higher alkaline phosphatase activity than the anterior lobe and hypothalamus. This high alkaline phosphatase activity in the posterior lobe is due to a concentration of the enzyme in the capillary walls of the lobe. Alkaline phosphatase in the capillary walls is more active in the posterior lobe than in the anterior and intermediate lobes. The posterior lobe has more densely arranged blood vessels than the two other lobes in the pituitary body. Alkaline phosphatase activity of the posterior lobe in the rat is not changed by dehydration or administration of corticoids. The relation between alkaline phosphatase activity in the posterior lobe and release of hormones into the circulation is discussed.

Recent histochemical studies have shown that there is a correlation between presence of alkaline phosphatase in cells and activity in transport of materials. Capillaries provide a most striking example of this (1). On the other hand, it has been demonstrated that a rapid discharge of antidiuretic hormone from posterior lobe to blood is elicited by stress (2). Therefore, it is likely that in the posterior lobe of the pituitary gland there must be an active movement of hormonal substances from nerve endings to blood vessels. The present experiments were designed to study the relation between the activity of alkaline phosphatase of the posterior lobe and release of hormones from the lobe into blood vessels in the rat. Furthermore, the enzyme activity was compared with that of the anterior lobe and hypothalamus.

MATERIAL AND METHODS

Female adult rats, weighing about 180 gm. were used for the study of characteristics and distribution of the alkaline phosphatase of the tissues employed. For the study of the localization of alkaline phosphatase, the tissues were treated essentially according to the method of Gomori (3). They were fixed in cold acetone for 2 hours. The sections were embedded in paraffin and cut at 6μ. The incubation time was 2 hours in the reaction mixture of pH 9.2–9.8. Control runs without substrate were made in parallel. To assay the enzyme activity, Ohmori's method was employed (4, 5). The tissues of hypothalamus, anterior lobe and posterior lobe including intermediate lobe were weighed on a torsion balance immediately after removal from the body. The homogenates were made to contain a particular concentration of the tissues per 1.0 ml. by adding an appropriate volume of cold de-ionized water. These procedures were carried out at 0–5° C. The homogenate of the posterior lobe included the intermediate lobe. The reaction tube containing 1.0 ml. of ammonium buffer and 1.0 ml. of the substrate, disodium *p*-nitrophenylphosphate (0.05%) was placed for at least 3 minutes in a water bath at 37° C and then 1.0 ml. of the homogenate of tissue was introduced into the tube to start the enzyme action. After the incubation for a fixed length of time at 37° C, with occasional shaking, 1.0 ml. of 20% trichloroacetic acid was added to stop the enzyme action. A 3.0 ml. aliquot was taken from the deproteinized supernatant after centrifugation and added to 3.0 ml. of saturated Na_2CO_3 solution. After some minutes, yellow colored *p*-nitrophenol in this mixture was determined by measuring the optical density at 430 mμ, using an electrophotometer. The activity of phosphatase is given as μg. *p*-nitrophenol liberated in the reaction mixture. To determine the pH optima, the enzyme activity was measured at intervals of 0.2 pH units from 8.4 to 10.4 (ammonium buffer). In these tests the homogenates were the following concentration: hypothalamus, 5.0 mg./cc; anterior lobe of pituitary 1.85 mg./cc; posterior lobe of pituitary 0.66 mg./cc. The incubation time at 37° C was 20, 30 or 40 minutes for hypothalmus, anterior lobe or posterior lobe, respectively. Four similar determinations were made. In another series of experiments, the effect of varying enzyme concentration on phosphatase activity was observed using the following different amounts of the homogenized tissues per cubic centimeter: hypothalmus, 1.0, 3.0 and 6.0 mg.; anterior lobe, 1.0, 1.5 and 3.0 mg.; posterior lobe, 0.4, 0.8 and 1.6 mg. The incubation time was 20 (hypothalamus), 30 (anterior lobe) or 40 minutes (posterior lobe) at 37° C at the optimal pH. The relation of the length of incubation (10, 30 and 40 minutes) to total enzymal activity was tested using tissue homogenates of 5.9 mg. of hypothalamus, 1.33 mg. of anterior lobe, or 0.44 mg. of posterior lobe per 1 ml. at 37° C at the optimal pH. After the separate determination of these enzyme characteristics, the alkaline phosphatase activity of the three tissues was compared under the following conditions in each of 6 animals. The homogenates of hypothalmus, anterior lobe and posterior lobe were prepared to contain 5.0, 1.5 and 0.5 mg. per 1 ml. and the incubation time at 37° C was 20, 40,

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and 40 minutes at the optimal pH, respectively. The relative enzymal activity of the three tissues was calculated from these simultaneous data. Control runs, blanks without added tissue, were always made in parallel with the experimental ones.

In dehydration experiments, two groups of littermate rats were used, with 6 adult females in each group. The body weights were between 160 and 195 gm. Three females of one litter, and three of another, did not receive water for 7 to 11 days, but were allowed to take food *ad libitum*. The 6 remaining rats were given food and water freely and served as controls. The alkaline phosphatase activity of the posterior lobe of each animal was determined at the optimal pH and 37° C. The incubation time was 40 minutes. The concentration of the tissue was 0.4–0.5 mg. per 1.0 ml. of the homogenate. In the next experiment, the effects of desoxycorticosterone acetate (DCA) and cortisone acetate on the activity of the enzyme were studied. Four adult female rats, weighing about 130 gm., received 5 injections of 5 mg. of DCA. The injections were made every other day and the animals were killed on the day after the last injection. Five other female rats, weighing about 130 gm. were given 5 mg. of cortisone acetate and treated in the same way as in the DCA experiment. Six untreated female rats served as controls. The alkaline phosphatase activity of the posterior lobe of each animal was assayed.

RESULTS

Deposits of cobalt sulfide, indicating alkaline phosphatase activity, were most dense in the capillary walls of posterior lobe. In fact, the reaction of the capillary walls was higher in the posterior lobe than in the anterior and intermediate lobes of pituitary body (Figs. 1 and 2). The figures show also more densely arranged blood vessels in the posterior lobe than in the anterior and intermediate lobes. There is at present no agreement regarding the presence of alkaline phosphatase in nuclei as revealed by the Gomori's method, however, using this technique, deposits of cobalt sulfide were found to be heavier in the nuclei of anterior lobe cells than in those of the posterior and intermediate lobes, and were not found in cytoplasm of the cells of three pituitary lobes, under the experimental conditions used.

The activity optimum of the phosphatase was around pH 10.0 for the hypothalamus, anterior lobe and posterior lobe including intermediate lobe. The activity of the enzyme in the three tissues was linear with respect to the enzyme concentration used, and had a linear relation to the incubation time between 10 and 40 minutes at pH 10.0 and 37° C.

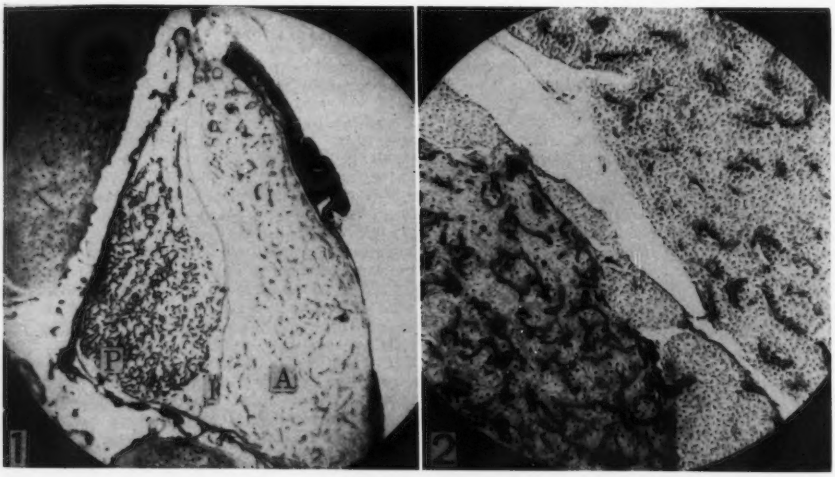

FIG. 1. Alkaline phosphatase in pituitary body of rat. Note strong reaction in capillary walls in posterior lobe. P, posterior lobe; I, intermediate lobe; A, anterior lobe, ×30.

FIG. 2. Higher magnification of Fig. 1. ×95.

The amounts of *p*-nitrophenol liberated in a period of 40 minutes by 1.0 mg. of each tissue at pH 10.0 and 37° C were 4.7 ± 0.40 μg. by hypothalamus, 2.0 ± 0.25 μg. by anterior lobe and 17.1 ± 0.76 μg. by posterior lobe. The figures represent the averages of 6 animals, and the respective standard errors. The alkaline phosphatase activity was higher in the posterior lobe than in the hypothalamus or anterior lobe. By neither dehydration, or administration of DCA or cortisone acetate was the enzymal activity of posterior lobe tissue changed (Table 1).

TABLE 1. EFFECTS OF DEHYDRATION AND CORTICOIDS ON ACTIVITY OF ALKALINE PHOSPHATASE IN POSTERIOR LOBE OF PITUITARY BODY OF RAT (pH 10.0, 37°C.)

Treatments	Number of animals	*p*-Nitrophenol (μg.) liberated in 40 minutes per 1 mg. of tissue
Dehydrated animals	6	19.8 ± 0.60*
Controls	6	18.7 ± 1.07
Animals injected with DCA	4	22.7 ± 2.45
Animals injected with cortisone acetate	5	21.4 ± 1.93
Controls	6	22.1 ± 1.49

* Standard error.

DISCUSSION

The high activity of alkaline phosphatase of the posterior lobe may be due chiefly to the enzyme activity in capillary walls since the histochemical study showed a high concentration of the enzyme in these sites. Considering the results of his stalk-section experiments, and the observations made by several other workers, Barrnett suggested that the blood vessels of the posterior lobe are permeable to large molecules such as vital dye proteinates or silver salts combined with protein, whereas brain capillaries are generally impermeable (6).

From these observations, it may be supposed that the characteristic high alkaline phosphatase activity of the walls of blood vessels may have a role in the permeability of the blood vessels to neurosecretory substances or posterior lobe hormones and to such proteinates as those discussed by Barrnett. But the activity of alkaline phosphatase in the posterior lobe of the rat was not changed by the following treatments, which may accelerate the release of posterior lobe hormones: dehydration or administration of either DCA or cortisone acetate. It would seem, therefore, that the activity of the enzyme in the capillary walls of the posterior lobe would not be a limiting factor in controlling the rate of release of neurohypophyseal hormones into the blood. It is not clear what the role of abundant alkaline phosphatase is in the capillary walls of posterior lobe. Rich vascularity in the posterior lobe has also been observed by Ferrer (7) using India ink. This vascularity may assure the rapid movement of the hormones from posterior lobe into the blood circulation.

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IMPROVED INTRAVENOUS GLUCOSE TOLERANCE IN THYROIDECTOMIZED OR HYPOPHYSECTOMIZED RATS TREATED WITH TRIIODOTHYRONINE[1]

ABSTRACT

Treatment with triiodothyronine improved or normalized the diminished intravenous glucose tolerance of thyroidectomized or hypophysectomized male rats.

Impaired disposal of intravenously injected glucose has been observed both in thyroidectomized (1, 2) and in hypophysectomized (3) rats. In the present study attempts to alleviate this diminished glucose tolerance by means of treatment with triiodothyronine have proved successful.

MATERIAL AND METHODS

Male Holtzman rats weighing approximately 200 gm. were surgically thyroidectomized under ether anesthesia. All rats that were used in this study had no macroscopically recognizable thyroid remnants at the time of necropsy, and were severely hypothyroid, as indicated by a reduced growth rate as well as (in the animals not treated with thyroid hormone) by the characteristic gelatinous appearance of the anterior hypophysial lobe (4) which is due to degranulation of the acidophils. Unoperated controls were either selected to match the final weight of the thyroidectomized animals (220–240 gm.) or were pair-fed with them. Hypophysectomized male Sprague-Dawley rats weighing 180–200 gm. were obtained 8 days after the operation from the Hormone Assay Laboratories, Chicago, Ill. All rats used had no hypophysial fragments detectable by inspection of the sella turcica and markedly atrophic testes. L-3,5,3′-triiodothyronine sodium (T_3) was dissolved in water made alkaline with NaOH and injected once a day subcutaneously in a volume of 1 ml. Dosages and schedules are given in Table 1. One group of thyroidectomized rats was subcutaneously injected with a single dose (1 mg./100 gm.) of somatotrophin (STH)[2] in a mildly alkaline solution 12 hours before the glucose tolerance test. All rats were fed Rockland diet and fasted 24 hours before the performance of the glucose tolerance test. Glucose (100 mg./100 gm. in 0.26 ml. of aqueous solution) was injected into the saphenous vein without anesthesia. One tenth ml. blood samples were obtained from the tail vein before and (at the intervals indicated in Table 1) after the injection. Blood sugar levels were determined by the Somogyi-Nelson method.

RESULTS

Table 1 summarizes the findings obtained. Thyroidectomized rats had variable fasting blood sugar levels which averaged higher than those of controls fed *ad libitum* before the final fast (Groups Ia+b vs. IV). Their glucose tolerance was clearly impaired in comparison with that of *ad libitum* fed-fasted or pair-fed-fasted (Group V) controls. This confirms earlier observations (1, 2). The fasting hyperglycemia and unimpaired glucose tolerance of the controls on the restricted regimen is also in agreement with

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[1] Aided by grant A-932, USPHS.

[2] STH was kindly provided by Dr. A. E. Wilhelmi on behalf of the National Institutes of Health.

TABLE 1. INTRAVENOUS GLUCOSE TOLERANCE OF THYROIDECTOMIZED OR HYPOPHYSECTOMIZED RATS

Group	No. rats	Operation	Test performed days postop.	Treatment	Dose/day	Treatment before test	Initial blood sugar	Blood sugar change†, minutes after i.v. glucose			
								10	30	60	120
								mg.%			
Ia(1)**+b	8	Thyrex	34–35	—	—	—	95 ±8*	+198 ±8	+174 ±8§	+128 ±11§	+ 58 ±7§
II	8	Thyrex	33–38	T_3	20 μg.	last 4 days	117 ±2	+142 ±4	+ 93 ±8	+ 40 ±5	− 7 ±5
III	4	Thyrex	35	STH	1 mg./100 gm.‡	12 hours	79 ±4	+211 ±3§	+202 ±4§	+154 ±7§	+ 57 ±7§
IV	8	—	—	—	—	—	78 ±2	+159 ±9	+104 ±10	+ 44 ±5	0 ±2
Ia(1)	3	Thyrex	35	—	—	—	74 ±2	+205 ±9	+189 ±9	+159 ±15	+117 ±34
Ia(2)	3	Thyrex	45	T_3	2 μg.	last 10 days	92 ±10	+194 ±3	+175 ±8	+121 ±12	+ 1 ±11
Ib	5	Thyrex	34	—	—	—	108 ±6	+193 ±13	+165 ±9§	+110 ±7§	+ 23 ±6
V	5	—	—	pair-fed with Ib	—	—	89 ±1	+170 ±3	+104 ±8	+ 39 ±4	− 1 ±4
VI	7	Hypex	24	—	—	—	60 ±2	+155 ±8	+125 ±8	+ 80 ±11	+ 22 ±9
VII	8	Hypex	24	T_3	2 μg.	last 10 days	65 ±3	+134 ±7	+ 80 ±7§	+ 36 ±7§	− 3 ±4

* Mean ±standard error.
† Compared with initial blood sugar.
‡ Single dose.
§ Difference from control significant at 1% level (control groups: IV, V, VI).
** Ia (1) and (2) refers to two determinations on the same group of rats.

previous findings (5). STH failed to alleviate the glucose intolerance of thyroidectomized rats (Group III). Treatment of 3 rats (Group Ia) with 2 μg. of T_3 daily for 10 days led to some elevation of fasting blood sugar levels and a definite improvement in glucose tolerance. Twenty μg. of T_3 given daily for 4 days caused uniform fasting hyperglycemia[3] and normalization of glucose tolerance (Group II). Hypophysectomized rats (Group VI) displayed the expected fasting hypoglycemia and the somewhat diminished glucose tolerance[4] previously described (3). The fasting blood sugar level was not significantly affected but the glucose tolerance was returned to normal by the daily injection of 2 μg. of T_3 for 10 days (Group VII).

DISCUSSION

The decreased glucose tolerance seen both in thyroidectomized and, to a lesser extent, in hypophysectomized rats could be due to hypothyroidism or conceivably to STH deficiency. STH is generally believed to be produced by the pituitary acidophils. The degranulated acidophils of thyroidectomized rats appear to contain and secrete subnormal amounts of STH (6, 7). STH increases the rate of removal of blood glucose in dogs (8) and in rats (9). Treatment with thyroid hormone not only abolishes the hypothyroidism of thyroidectomized rats but also regranulates their pituitary acidophils (3) and hence presumably restores STH secretion. However, under the conditions of our experiment STH failed to influence the poor glucose tolerance of thyroidectomized rats. Further, the improved glucose tolerance which T_3 brought about in hypophysectomized rats obviously could not be ascribed to any effect mediated through the hypophysis. It is therefore simplest to postulate that the decreased glucose tolerance which characterizes both the thyroidectomized and the hypophysectomized state is due to the hypothyroidism common to both conditions. It may be caused by thyroid hormone deficiency *per se*, or by concomitant changes such as the lowered metabolic rate. Further studies will also have to show whether any specific pathway of disposal of excess glucose is preferentially or exclusively affected, and if so, whether thyroid hormone restores normal glucose tolerance by improving the removal of blood glucose along the same pathway.

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[3] The most likely reason for the hyperglycemia due to T_3 is enhanced gluconeogenesis from protein. The paradox of fasting hyperglycemia and normal glucose tolerance may indicate that different mechanisms are involved in the disposal of a sudden excess of exogenous glucose and the maintenance of normal blood sugar levels in the face of a sustained increase of glucose output by the liver.

[4] Cf. blood sugar excess at 60 minutes after glucose injection in group VI, Table 1, with values in untreated groups IV and V ($p<0.01$).

[5] Medical student summer research fellow.

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THE ACTIVITY OF SYNTHETIC ISOESTRONES IN PYRIDINE NUCLEOTIDE TRANSHYDROGENATION[1]

ABSTRACT

Synthetic isoestrones were examined for the capacity to mediate pyridine nulceotide transhydrogenation or to act as substrates for 17β hydroxy-steroid dehydrogenase. 8-isoestrone was active in both systems. The other isoestrones tested showed neither activity.

Previous studies have indicated that certain steroids have the property of accelerating transhydrogenation from a catalytic amount of TPNH to DPN in the presence of placental homogenate (1, 2). The structural requirements for striking stimulation of this system are the presence of an aromatic ring A and a 17B hydroxyl or 17 keto group (3, 4). The existence of synthetic isomers of estrone (5) permitted study of other steric requirements.

METHODS

The preparation of the placental homogenate and the assay system for measuring steroid coenzyme activity in terms of additional ketoglutarate production from isocitrate by placental homogenate have been described (4). For the measurement of 17β hydroxy-steroid dehydrogenase at low steroid levels it was necessary to purify the placental homogenate partially, by precipitating the activity at 50% saturation with ammonium sulfate and dialyzing the redissolved protein against 0.01 *N* phosphate buffer, pH 7.0.

The action of the 17β hydroxy-steroid dehydrogenase (6) was measured by the direct fluorimetric estimation of DPNH in an Aminco-Bowman Spectrofluorimeter. The substrate mixture consisted of phosphate buffer 0.006 *M*, pH 6.8, $MgCl_2$ 0.006 *M*, Versene 0.001 *M*, 0.01 ml. of propylene glycol containing 0.003 μ-moles of the appropriate steroid, and 50 μg. enzyme in a total volume of 1.0 ml. The reaction was started by addition of 0.005 μ-moles of DPNH to the substrate mixture at room temperature.

RESULTS

Table 1 shows that of the 5 synthetic isoestrones[2] tested only dl-8-isoestrone showed significant ability to increase the rate of transhydrogenation from TPNH to DPN as shown by increased α-ketoglutarate formation. In the 17β hydroxy-steroid dehydro-

Table 1. Effect of isoestrones on placental transhydrogenase

Compound	$3.6\times10^{-7}M$	$3.6\times10^{-6}M$	$3.6\times10^{-5}M$
estradiol-17β	0.116	0.122	0.126
estrone	0.076	0.117	0.120
dl-8-isoestrone	0.044	0.112	0.097
dl-9-iso-13-isoestrone	0.001	0.003	0.004
dl-13-isoestrone	0.0	0.0	0.0
dl-9-isoestrone	0.0	0.0	0.0
dl-14-isoestrone	0.0	0.0	0.0

Each value represents the mean obtained from three experiments. Triplicate determinations in each experiment agreed within 5%. The results are expressed in micromoles of ketoglutarate produced in 1 hour at 30° due to the steroid.

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[1] This work was supported by United States Public Health Service Grant CY-3225-C1.

[2] The synthetic isoestrones used in this study were the very kind gift of Professor W. S. Johnson, Department of Chemistry, University of Wisconsin.

TABLE 2. INHIBITION OF ESTRADIOL INDUCED TRANSHYDROGENATION

Compound	$3.6\times10^{-5}M$	$3.0\times10^{-6}M$
dl-13-isoestrone	84	23
dl-9-iso-13-isoestrone	56.5	14
dl-9-isoestrone	62	14
dl-14-isoestrone	77.3	16.5

The results are expressed as the per cent inhibition of ketoglutarate production by $3.6\times10^{-6}M$ estradiol-17β in 1 hour at 30°C.

genase system the only isoestrone with activity was dl-8-isoestrone. This substance was reduced at the same rate as estrone, 1.8×10^{-4} μ-moles per minute.

The four inactive isoestrones were found to inhibit estradiol-17β potentiation of α-ketoglutarate by the placental pyridine nucleotide transhydrogenation system (Table 2).

DISCUSSION

The activity of 8-isoestrone and inactivity of the other isoestrones at the level tested in both the 17β hydroxy-steroid dehydrogenase and pyridine nucleotide transhydrogenation systems is consistent with the mechanism proposed by Talalay and Williams-Ashman that transhydrogenation is effected by the dehydrogenase capable of reaction with both pyridine nucleotides (2). The oxidation of 8-isoestrone at higher substrate levels by 17β hydroxy-steroid dehydrogenase has been observed by Langer (7). The data presented are consistent with the need for a reactant to approach the steroid from the alpha side along a flat portion of the steroid molecule comprising rings C and D. Munck, Scott, and Engel concluded that this portion of the molecule was involved in purine binding (8). Marcus and Talalay studied the substrate specificity of a 17β hydroxy-steroid dehydrogenase-DPN and found that the kinetic data were consistent with interaction of the enzyme system with the α side of the steroid molecule and positions 3 and 17 (9).

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[3] United States Public Health Postgraduate Fellow.

The Endocrine Society

1959 PROGRAM OF THE MEETING OF THE ENDOCRINE SOCIETY IN ATLANTIC CITY

THURSDAY, JUNE 4, 1959

8:00 A.M.—Registration—

I. 9:00 A.M.—THYROID

Carolina Room—Chalfonte

Chairmen: *J. E. Rall and W. Parson*

1. The Effect of Trypan Blue on Thyroid Function in the Rat.
   Takashi Yamada (Introduced by Monte A. Greer).
2. The Effect of Excessive Iodide on the Thyroid of the Rat.
   Valerie A. Galton and *Rosalind Pitt-Rivers.* (Introduced by Sidney Ingbar).
3. Comparison of in Vivo and in Vitro Tissue Effects of Triiodothyronine.
   Roger L. Greif.
4. The Effect of Triiodothyropropionic Acid on Serum Cholesterol and Oxygen Consumption in Myxedema and Euthyroidism.
   Robert D. Leeper (by invitation), *Richard S. Benua, Soichi Kumacka* (by invitation), *Jacob L. Brener* (by invitation) and *Rulon W. Rawson.*
5. Respiratory Stimulation in Young Dogfish by Thyroid Hormone.
   Aubrey Gorbman and *A. W. Pritchard* (by invitation). (Invited paper—20 minutes.)

INTERMISSION

6. Idiopathic Familial Elevation of Thyroxine Binding Protein.
   William H. Beierwaltes.
7. Metabolism of Thyroxine by Human Tissue Slices.
   Edwin C. Albright and *Frank C. Larson* (by invitation).
8. Metabolic Regulation of Organic Iodinations in Thyroid Homogenates.
   George C. Schussler (by invitation) and *Sidney H. Ingbar.*
9. Protein Iodination in Sheep Thyroid Mitochondrial-Microsomal Preparations.
   Leslie J. DeGroot and *Evelyn Carvalho* (introduced by John B. Stanbury).
10. Persistent Thyroid Function After Hypophysectomy.
    O. H. Pearson, A. G. Pazianos (by invitation), *E. Greenberg* (by invitation), and *R. S. Benua.*
11. Effect of Stimulation of the Cervical Sympathetic Nerve on the Thyroidal Release of I^{131}-Labeled Hormones.
    Shiro Iino, Kazuo Shizume and *Shigeo Okinaka* (introduced by Monte A. Greer).

II. 2:00 P.M.—ADRENAL CORTICAL SYNDROMES—STEROID METABOLISM

Carolina Room—Chalfonte

Chairmen: *J. McArthur and A. Bongiovanni*

12. 6 Beta Hydroxylation of Cortisol, a Quantitatively Important Pathway of Metabolism in the Human Newborn.
    E. Colle (by invitation), *R. A. Ulstrom, J. Burley* (by invitation) and *R. Gunville* (by invitation).

13. The Urinary Excretion of Three C-21 Steroids in the Adrenogenital Syndrome.
Alfred M. Bongiovanni, Walter R. Eberlein, and *J. Darrel Smith* (by invitation).
14. The Renal Handling of 17-Hydroxycorticosteroids.
R. O. Morgan (by invitation), *A. J. Blair* (by invitation) and *J. C. Beck.*
15. The Influence of ACTH and Hypophysectomy on the Steroid Conjugating Capacity of the Liver.
David L. Berliner (by invitation), *Charles J. Nabors, Jr.* and *Thomas F. Dougherty.*
16. Direct and Indirect Estimation of Pituitary ACTH Reserves in Man Following Steroid Therapy
Donald A. Holub (by invitation), *Julian I. Kitay, Andrew G. Frantz* (by invitation) and *Joseph W. Jailer.*
17. ACTH Suppression Tests in Patients with Cushing's Syndrome.
Grant W. Liddle.

INTERMISSION

18. Urinary Steroid Metabolites in Hypophysectomized Women During Adrenal Insufficiency and Initial Phases of ACTH Stimulation.
Hildegard Wilson, Mortimer B. Lipsett, and *Lillian C. Butler* (by invitation).
19. Suppression of Urinary 17-Ketosteroids in Virilized Women by Estrogen Therapy.
Raymond C. Mellinger and *Richmond W. Smith, Jr.*
20. Transcortin: Characteristics and Levels in Various Conditions.
Avery A. Sandberg and *W. Roy Slaunwhite, Jr.*
21. Studies on the Mechanism of Elevation of Plasma 17-Hydroxycorticosteroids Induced by Estrogen Administration in Man
Eleanor Z. Wallace and *Anne C. Carter.*
22. Endocrine Effects of Methylenedianiline.
William W. Tullner.
23. Salt Losing Hormone in Hyperkalemic Familial Periodic Paralysis.
R. Klein.

III. 2:00 P.M.—PARATHYROIDS: PITUITARY ADRENAL SYSTEM

Viking Room—Haddon Hall

Chairmen: *G. D. Whedon and D. Nelson*

24. Isolation of Parathyroid Hormone Following Extraction with Phenol.
G. D. Aurbach.
25. Purification of Parathormone B by Countercurrent Distribution.
Howard Rasmussen.
26. Activation of Parathyroid Hormone by Cysteine.
Alexander D. Kenny.
27. Effect of Parathyroid Extract on Bone Matrix and Seromucoid.
William P. Deiss and *Leila B. Holmes* (by invitation).
28. Further Evidence for a Dual Effect of Parathyroid Hormone.
John J. Canary (by invitation), *H. Allan Bloomer* (by invitation), *Gabriel G. Carreon* (by invitation) and *Laurence H. Kyle.*
29. ACTH Secretion in Rats with Hypothalamic Lesions.
A. Brodish.

INTERMISSION

30. Limbic System Influences on the Pituitary-Adrenal Cortical System.
John W. Mason, Walle J. H. Nauta (by invitation), *Joseph V. Brady* (by invitation), and *James A. Robinson* (by invitation).

31. Effect of Diencephalic Stimulation on Adrenocortical and Adrenal Medullary Secretion in the Dog.
William H. Ganong and *Alan Goldfien.*
32. Adrenal Cortical and Medullary Responses to Trauma in Dogs with Isolated Pituitaries.
Richard H. Egdahl (introduced by D. M. Hume).
33. The Early and Late Effects of Hypothalamic Destruction or Removal on Pituitary-Adrenal Activity.
David M. Hume and *Benjamin F. Jackson* (by invitation).
34. Ion-Exchange Chromatography of Pituitary Preparations.
Andrew V. Schally (by invitation) and *Roger Guillemin.*
35. ACTH Releasing Factor Active in the Guinea Pig Test.
M. Sideman (by invitation) and H. Sobel.

FRIDAY, JUNE 5, 1959

8:30 A.M.—Registration—

IV. 9:00 A.M.—ANTERIOR PITUITARY

Carolina Room—Chalfonte

Chairmen: *E. Knobil and F. L. Engel*

36. The Cells of the Human Adenohypophysis in Adrenal Cortical and Thyroid Disorders.
Calvin Ezrin and *Heidi Swanson* (introduced by R. F. Farquharson).
37. A Method for the Quantitative Biological Assay of ACTH in Blood.
Paul L. Munson, Wolfgang Toepel (by invitation) and *Mindel C. Sheps.*
38. Increase in Sugar Permeability in the Rat Adrenal Produced by ACTH.
J. Eichhorn, I. D. K. Halkerston, M. Feinstein, amd *O. Hechter* (by invitation).
39. Contrasting Requirements for the Lipolytic Action of Corticotropin and Epinephrine on Adipose Tissue in Vitro.
J. Earle White (by invitation), *Enrique Lopez* (by invitation) and *Frank L. Engel.*
40. Evidence for Species Specificity of Human, Beef and Rat ACTH Demonstrated by Assay in Vitro.
Julian I. Kitay, Donald A. Holub (by invitation) and *Joseph W. Jailer.*
41. A Comparative Immunological Investigation of Pituitary Growth Hormone from Various Species.
T. Hayashida (by invitation) and *C. H. Li.*

INTERMISSION

42. Growth-Stimulating and Other Effects of Human Growth Hormone (Li) in a Pituitary Dwarf.
Roberto F. Escamilla, John J. Hutchings (by invitation), *William C. Deamer* (by invitation) and *Choh Hao Li.*
43. Endocrine Function in Acromegaly.
George J. Hamwi and *Thomas G. Skillman* (by invitation).
44. The Influence of Hypophysectomy and Growth Hormone on Oxidation of Palmitate-1-C^{14} by the Rat.
M. J. Franklin (by invitation) and *E. Knobil.*
45. Unesterified Fatty Acid Oxidation Rates in Acromegaly, Myxedema, Hyperthyroidism and Diabetes.
Josiah Brown and *Leslie R. Bennett* (by invitation).
46. Evidence for Direct Action of Growth Hormone (GH) on Red Cell Formation.
Albert S. Gordon, Burton S. Dornfest (by invitation) and *Anthony Crusco.*

47. Factors Involved in Production of Suspensions of Free Cells Derived from Hypophyses of Rats.
Griff T. Ross (by invitation) and *Robert C. Bahn.*

1:30 P.M.—ANNUAL BUSINESS MEETING, Carolina Room, Chalfonte

V. 2:30 P.M.—ESTROGENS AND ANDROGENS

Carolina Room, Chalfonte

Chairmen: *R. Huseby and L. L. Engel*

48. The Isolation and Identification of Estrogens from Peripheral Blood with a Practical Method of Analysis.
Georg W. Oertel (by invitation), *Charles D. West,* and *Kristen B. Eik-Nes.*
49. "Free Estriol" in Blood Plasma.
Joseph C. Touchstone and *John W. Greene, Jr.* (by invitation).
50. Effect of Chorionic Gonadotropin (HCG) on Conversion of Estradiol to Estriol by Perfused Human Placenta.
Philip Troen.
51. Physiologic and Biochemical Differences between the Estrogen-Sensitive Transhydrogenase and Estradiol Dehydrogenase.
C. A. Villee, D. D. Hagerman (by invitation) and *J. M. Spencer* (by invitation).
52. The Inhibition of Placental Steroid Mediated Transhydrogenase by 2′ Adenylic Acid.
Nina Hollander (by invitation), *Joseph D. Brown* (by invitation) and *Vincent P. Hollander.*
53. Adrenal Estrogens: In Vivo Conversion of Cortisone to Phenolic Estrogens.
Edward Chang (by invitation) and *Thomas L. Dao.*

INTERMISSION

54. Estrogen Synthesis in a Male Patient with a Feminizing Adrenocortical Carcinoma and Hypertension.
C. D. West, L. F. Kumagai (by invitation), *E. L. Simons* (by invitation) and *P. E. Wall* (by invitation).
55. The Effect of Estrogen on Steroid Levels and Urine.
W. L. Harrmann, I. Schindl (by invitation), and *P. K. Bondy.*
56. The Conversion of Progesterone-4-C^{14} to 21-Hydroxylated Steroids by Normal Rat Testis Stimulated with Human Chorionic Gonadotropin.
Hernan F. Acevedo (by invitation), *Oscar V. Dominguez* and *Leo T. Samuels.*
57. Steroid 21-Hydroxylase in Mouse Testicular Interstitial Cell Tumors.
Oscar V. Dominguez, Robert A. Husby and *Leo T. Samuels.*
58. Studies of Structure-Function Relationships of Steroids: Anabolic Potency of Some Testosterone Analogs.
Herbert A. Burke, Jr. (by invitation) and *Grant W. Liddle.*
59. Influence of Androgen on Prostatic Protein Synthesis.
Wells E. Farnsworth (introduced by Grosvenor W. Bissell).

VI. 2:30 P.M.—PITUITARY HORMONES—NEUROENDOCRINE MECHANISM

Viking Room, Haddon Hall

Chairmen: *J. Everett and A. Gorbman*

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

60. Lithosperm Inhibition of Blood Pressure Depressor Effect of Oxytocin in the Fowl.

W. R. Breneman, Marvin Carmack, Daniel Overack and *Richard Shaw* (introduced by Aubrey Gorbman).

61. Plasma and Pituitary LH: Detection and Quantitative Bioassay in the Rat Following Gonadectomy.
Albert Parlow (introduced by R. O. Creep).
62. Use of the Red Eft Diamictylus Viridescens in the Assay of Prolactin.
William C. Grant, Jr. (introduced by Aubrey Gorbman).
63. Effects of Captivity on Production of Gonadotrophins in Pintail and Black Ducks and Response to Exogenous Hormones.
R. E. Phillips and *A. van Tienhoven* (introduced by S. L. Leonard).
64. Ovulation in the Domestic Fowl.
R. D. Fraps. (Invited paper, 20 minutes).

INTERMISSION

65. Ovulation After Stimulating the Rat Hypothalamus Late in Diestrus.
J. W. Everett, R. L. Riley (by invitation) and *C. D. Christian* (by invitation).
66. Development of Permanent Abnormal Cycles of Behavior in Rats After Experimental Pituitary Damage.
Curt P. Richter.
67. Hypothalamus and Growth.
S. Reichlin.
68. TSH "Rebound Phenomenon" in Rats After Goitrogen Withdrawal.
S. A. D'Angelo.
69. Pituitary Hormone Production and Release in the Thyroidectomized Rat After Thyroxin Administration.
A. N. Contopoulos, M. E. Simpson (by invitation) and *A. A. Koneff* (by invitation).
70. Subcortical Regions of the Primate Brain Regulating Release of Vasopressin from the Neurohypophysis.
Joseph F. Dingman, Eduardo Gaitan (by invitation), *Akira Arimura* (by invitation) and *Robert G. Heath* (by invitation).

VII. ANNUAL DINNER, Haddon Hall Hotel

E. Perry McCullagh, President, presiding

6:30 P.M.—Cocktails, The English Lounge
Sponsored by the donors of the Awards of the Society:
Ciba, Schering, Ayerst, Upjohn and Squibb. Attendance limited to holders of dinner tickets.

7:30 P.M.—Dinner—The Vernon Room
Presentation of the 1959 Awards of the Society by Charles Kochakian:
The Fred Conrad Koch Award
The Ciba Award
Ayerst Fellowship
Schering Scholar
Upjohn Scholar
President's Address: Dr. McCullagh

SATURDAY, JUNE 6, 1959

8:30 A.M.—Registration—Stair Hall, Haddon Hall Hotel

9:00 A.M.—OPENING SCIENTIFIC SESSION OF THE AMERICAN DIABETES ASSOCIATION WILL BE HELD AT THE HADDON HALL HOTEL IN THE VERNON ROOM. ENDOCRINE SOCIETY BADGE WILL ADMIT YOU TO THIS MEETING.

VIII. 9:00 A.M.—ADRENAL GLAND

Carolina Room, Chalfonte

Chairmen: *M. Grumbach and F. C. Bartter*

71. Stimulatory Effect of Aldosterone and Cortisol on the Work of the Isolated Rat Heart-Lung.
Kathyrn W. Ballard (by invitation) and *George Sayers.*
72. The Hemodynamic Parameter Mediating Increase in Aldosterone Secretion.
Donald S. Gann (by invitation), *Ivor H. Mills* (by invitation) and *Frederic C. Bartter.*
73. Preliminary Characterization of Glomerulotropin.
Gordon Farrell, Avery D. Pratt, Jr. (by invitation), *Laurence S. Harris* (by invitation) and *Robert C. Banks* (by invitation).
74. Changes in Metabolic Activities of Steroid Hormones Associated with Methylation and Hydroxylation in Ring D.
Maurice M. Pechet, Evelyn L. Carroll (by invitation) and *Anne P. Forbes.*
75. Effect of Walker Carcinoma on Steroid Diabetes in Rat.
David J. Ingle (introduced by Dwight J. Ingle).
76. Acute Effects of Adrenalectomy and Hydrocortisone on Glucose Tolerance of Diabetic and Eviscerate Rats.
O. K. Smith and *C. N. H. Long.*

INTERMISSION

77. Influence of Age, Adrenalectomy and Cortisol Administration on the Activity of Hepatic Glutamic-Pyruvic Transaminase.
Homer Harding, Fred Rosen and *Charles Nichol* (introduced by Avery A. Sandberg).
78. The Metabolism of 2 C^{14} Pyruvate in Normal and Adrenalectomized Rats.
William W. Winternitz.
79. Observations on the Role of the Adrenal Gland in the Metabolic Response to Surgery.
Maureen Smith, H. A. F. Dudley, J. S. Robson and *C. P. Stewart* (introduced by Grant W. Liddle).
80. Renal-Cardiovascular Pathology in Aging Rats With and Without Exposure to ACTH and Stressors.
George F. Wilgram (by invitation) and *Dwight J. Ingle.*
81. The Effects of Anoxis on Adrenal Cortical and Medullary Secretion in the Dog.
Alan Goldfien and *William F. Ganong.*
82. Effect of Adrenalectomy and Cortisone on Rate of Eruption of Maxillary Incisors in Female Rats.
L. V. Domm, W. A. Wellband (by invitation) and *J. P. Howard* (by invitation).

IX. 2:00 P.M.—INSULIN AND DIABETES

Carolina Room, Chalfonte

Chairmen: *S. Fajans and A. Renold*

83. Differential Resin Binding of Insulin in Sera from Insulin Responsive and Resistant Diabetic Subjects.
Marvin L. Mitchell and *Mary E. O'Rourke* (by invitation).
84. Serum Insulin-Like Activity in Patients with Pancreatic Islet-Cell Tumors.
Yves M. Dagenais (by invitation), *Albert E. Renold, Donald B. Martin* (by invitation) and *Vilma Lauris* (by invitation).

85. Role of Individual Organs in Carbohydrate Metabolism in Intact Normal Dogs.
Dorothy H. Henneman, William Shoemaker (by invitation) and *William Walker* (by invitation).
86. Hepatic Glucose Metabolism in Two Forms of Spontaneous Hyperglycemia.
Gaston R. Zahnd, Jean Christophe and *George Yerganian* (introduced by Albert E. Renold).
87. Carbohydrate Metabolism in Amphibians and Reptiles.
Malcolm R. Miller (Introduced by Frank L. Engel). (Invited paper, 20 minutes.)

INTERMISSION

88. On the Mechanism of the Blood Glucose Lowering Action of Insulin.
N. Altszular, A. Dunn (by invitation), *R. Steele, D. T. Armstrong* (by invitation), *J. S. Bishop* (by invitation) and *R. C. de Bodo.*
89. An Insulin Effect on Peptide Synthesis Independent of Glucose or Amino Acid Transport.
Ira G. Wool and *M. E. Krahl* (by invitation).
90. Comparison of Epinephrine and Insulin on Adipose Tissue Metabolism.
George F. Cahill, Jr., Bernard Leboeuf (by invitation), and *Robert Flinn* (by invitation).
91. The Action of Insulin, Tolbutamide and Hypoglycin A on the Adipose Tissue of Rats.
Thomas C. Smith, Lena Will (by invitation), and *Ira Ringler.*
92. Relation of Insulinogenesis to Pancreatic B Cell Acid and Glucose-6-Phosphatase.
Sydney S. Lazarus (introduced by Bruno W. Volk).
93. Effect of Glucagon on Hepatic Blood Flow and Metabolism in Unanesthetized Dog.
W. C. Shoemaker and *T. B. Van Itallia* (introduced by Jay J. Gold).

TO BE READ BY TITLE

See Abstracts 94–170

GORDON RESEARCH CONFERENCE

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

13–17 July 1959
Kimball Union Academy
Meriden, New Hampshire

W. D. Armstrong, Chairman
B. B. Migicovsky, Vice Chairman

13 July

Invited Short Communications
Felix Bronner, Chairman
Bone as a Mechanical Tissue
Jonathan Cohen, Chairman
Bone as a Problem in Mechanical Engineering

14 July

F. G. Evans. The Relation of the Microscopic Structure of Bone to Its Strength Characteristics and Other Physical Properties

H. Hollingshaus. Mechanical Properties of Bone From Radium Poisoned Dogs
W. T. Dempster. The Distribution of Osseous Material in the Mandible and Humerus, and Its Mechanical Importance
M. G. Hardinge. The Transmission of Weight Through the Knee Joint

Fluoride Metabolism
Harold C. Hodge, Chairman
D. A. Greenwood. Broad Aspects of Fluoride Metabolism
Yngve Ericsson. Radiofluoride Studies

15 July

P. H. Phillips. Bone Retention of Fluoride
C. S. Hobbs. Radiofluoride Distribution in Animals Under Chronic Fluoride Exposure
E. J. Largent. Urinary Fluoride Levels in Relation to Change in Osseous Radiopacity
F. J. McClure. Fluoride Content and the Chemistry of Human Skeletal Tissue
C. H. Carlson. Mechanisms of Renal Excretion of Fluoride Determined with Radiofluoride
Leon Singer. Plasma Fluoride Concentrations in Relation to Fluoride Intake

New Developments in Methodology in Study of Calcified Tissues
D. Dziewiatkowski, Chairman
Diego Carlstrom. Some New Aspects of Ultrastructure of Bone

16 July

James Irving. Histochemical Changes at Sites of Calcification
Richard Greulich. High Resolution Microradiography
Harald Schraer. Quantitative Determination of Bone Mineral Content and Bone Density In Vivo From Standardized Roentgenograms

Pathological Physiology of Bone and Calcium Metabolism
Franklin C. McLean, Chairman
G. Fanconi. Idiopathic Hypercalcemia

17 July

Albert Dorfman. Studies on Acid Mucopolysaccharides
Donald Fraser. Hypophosphatasia
Karl M. Wilbur. Mechanisms of Mineralization in Molluscs

Attendance. Requests for attendance at the conferences, or for additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From 15 June to 4 September 1959 mail should be addressed to Colby Junior College, New London, New Hampshire.

Registration and Reservations. Attendance at the Conferences is by application. Individuals interested in attending the conferences are requested to send their applications to the Director at least two months prior to the date of the Conference. *All applications must be submitted on the standard application form which may be obtained by writing to the office of the Director.* This procedure is important because certain specific information is required in order that a fair and equitable decision on the application may be made. Attendance at each Conference is limited to approximately 100 individuals.